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(54) **Methods and compositions for obtaining mature dendritic cells**

(57) We describe an improved method for generating sizable numbers of mature dendritic cells from non-proliferating progenitors in human blood. The first step or "priming" phase is a culture of T cell depleted mononuclear cells in medium supplemented with GM-CSF and IL-4 to produce immature dendritic cells. The second step or "differentiation" phase requires the exposure to dendritic cell maturation factor such as monocyte conditioned medium. Using this two-step approach, substantial yields are obtained. The dendritic cells derive from this method have all the features of mature cells. They include a stellate cell shape, nonadherence to plastic, and very strong T cell stimulatory activity. The mature dendritic cells produced according to this invention are useful for activating T cells.

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## Description

[0001] This invention was made with United States Government support under NIH grants AR-39552, AR-42557 and AI-24775. The United States Government has certain rights in this invention.

### TECHNICAL FIELD OF THE INVENTION

[0002] This invention relates to methods and compositions useful for activating cells of the immune system. The methods and compositions provided by this invention are useful for causing the maturation of non-proliferating immature dendritic cells to mature dendritic cells capable of processing and presenting antigen. This invention also relates to culture mediums which promote maturation of immature dendritic cells to mature dendritic cells. In addition, this invention relates to assays useful for detecting the presence in a test substance of a dendritic cell maturation factor.

### BACKGROUND OF THE INVENTION

[0003] Dendritic cells are specialized antigen presenting cells, critical for eliciting T cell mediated immune responses (Steinman, 1991; Caux et al. 1995a; Hart and McKenzie, 1990; Austyn, 1987). These specialized antigen presenting cells elicit both CD4+ helper cells (Inaba et al. 1990a; Inaba et al. 1990b; Crowley et al. 1990) and CD8+ killer cells (Por-gador and Gilboa, 1995; Mayordomo et al. 1995; Zitvogel et al. 1995) *in vivo*.

[0004] Because of the potent activity of dendritic cells to activate T cells, the art, e.g. Flanagan et al., *Env. J. Immunol.*, 24:605-610, 1994, has accepted the characterization of dendritic cells as "nature's adjuvant". It is therefore desirable to be able to use dendritic cells to process and present protein antigens as a means of modulating an individual's immune response, and in particular to activate an individual's T cells in connection with the treatment or prevention of disease.

[0005] The use of primed dendritic cells to activate cytotoxic T cells has been reported. For example, Paglia et al., recently reported that murine dendritic cells cultured from bone marrow precursors and exposed to antigen *in vitro* provide effective resistance to challenge with live tumor cells. Paglia et al., "Murine Dendritic Cells Loaded In Vitro With Soluble Protein Prime Cytotoxic T Lymphocytes Against Tumor Antigen In Vivop", *J. Exp. Med.*, 183:317-322 (1996).

[0006] The *in vitro* observations and the murine results have recently been extended to the treatment of humans with B cell lymphoma using mature dendritic cells which were primed *in vitro* with tumor antigens. Hsu et al., "Vaccination of Patients With B-cell Lymphoma Using Autologous Antigen-Pulsed Dendritic Cells", *Nature Med.*, 2:52-58 (1996). According to this report, the antigen used to prime the dendritic cells was idiotype antibody obtained from hybridomas made from the fusion of lymph node tumor cells with a mouse cell line. All of the four patients involved in this study exhibited significant antitumor idiotype PBMC proliferative responses. Positive clinical responses were also observed including one patient who experienced complete tumor regression. Hsu et al., however, express uncertainty whether dendritic cells obtained from expansion of dendritic cell cultures in the presence of GM-CSF, TNF- $\alpha$  or IL-4 would function equivalently in their ability to process and present antigen or to stimulate cellular immune responses as the freshly isolated cells.

[0007] Prior studies have identified proliferating dendritic cell progenitors within the small CD34+ subfraction of cells in human blood (Inaba et al. 1992; Caux et al. 1994; Caux et al. 1992; Caux et al. 1995). Methods have been developed for expanding these proliferating cells in culture to obtain sufficient numbers of cells to be useful for priming with antigen and administering to an individual to activate an immune response. Steinman et al. International patent application WO 93/20185. These dendritic cells can be stimulated with cytokines, particularly GM-CSF and, optionally TNF $\alpha$  or other cytokines, to develop into potent dendritic cells over 1-2 weeks in culture (Caux et al. 1992; Inaba et al. (1992)). Although useful dendritic cells can be produced from the proliferating precursors, it is desirable to obtain alternative methods of obtaining suitable numbers of mature dendritic cells for therapeutic purposes where proliferating progenitors are infrequent.

[0008] The removal of monocytes and lymphocytes from human blood has uncovered a small population of nonproliferating progenitors that require cytokines to develop into typical dendritic cells. These progenitor cells exist at a concentration of at most about  $10^6$  cells per 450-500 ml of blood (O'Doherty et al. 1994; O'Doherty et al. 1993; Thomas et al. 1993). More recently, the combination of GM-CSF and IL-4 has been shown to facilitate the generation of significantly larger numbers of dendritic cells from adherent blood mononuclear fractions, about  $3-8 \times 10^6$  per 40 ml of blood (Romani et al. 1994; Sallusto and Lanzavecchia, 1994 both of which are incorporated herein by reference). However, we have now determined that when the cytokines are removed, the cells revert to an adherent and less stimulatory state, that is, they do not have the properties of mature, stable dendritic cells. If the latter reversion were to take place *in vivo* during adoptive immunotherapy, the cells could be ineffective as adjuvants. In addition, it is desirable to develop a culture system independent of fetal calf serum.

[0009] Engleman et al. International Patent Application WO 95/34638 refers to a method of activating an immune response in a human patient by administering to the patient dendritic cells primed with antigen. Engleman et al. how-

ever refer to using dendritic cells isolated from the individual which exist in small numbers. Accordingly, it would be desirable to be able to obtain dendritic cells from a large population of cells present in an easily accessible tissue such as blood.

**[0010]** Two antigens, have recently been reported which, in addition to other antigens or phenotypic characteristics, distinguish mature dendritic cells from other types of white blood cells. Zhou and Tedder (Zhou and Tedder, 1995) reported that CD83, a member of the immunoglobulin superfamily that was cloned from an EBV induced B cell library, is expressed on dendritic cells in blood cells that were cultured for 2 days. This culture period is sufficient to allow a small subset of immature dendritic cells to mature (O'Doherty et al. 1993). CD83 has also been detected on some presumptive dendritic cells in the T cell areas of lymphoid organs, and on some B cells in germinal centers (Zhou et al. 1992). Langhoff and coworkers found that p55, an actin bundling protein, also marks the dendritic cells that are found in 2 day cultures of human blood (Mosialos et al. 1995). p55 is an intracellular protein that was discovered as an EBV induced host cell product. It is expressed by interdigitating cells and at high levels in the brain (Mosialos et al. 1995).

## SUMMARY OF THE INVENTION

**[0011]** This invention provides methods of preparing large numbers of stable, mature dendritic cells. The stable mature dendritic cells provided by this invention retain characteristics of a mature phenotype, including expression of dendritic cell markers p55 and CD83 and high levels of accessory molecules like CD86 and CD40, even when removed from contact with cytokines which promote the maturation of pluripotential peripheral blood mononuclear cells (PBMCs) to non-stable immature dendritic cells. As used herein, "stable" refers to retention of a mature dendritic cell phenotype for three days when mature dendritic cells are cultured in the absence of cytokines. Dendritic cells are considered "mature" according to this invention when they have an increased expression of one or more phenotypic markers, such as for example non-adherence to plastic and CD86 or other antigen markers, associated with the accessory function of dendritic cells following contact with a dendritic cell maturation factor.

**[0012]** In one embodiment of the invention, mature dendritic cells are produced in vivo or in vitro from immature dendritic cells derived from PBMC pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics. The method, according to the invention, comprises contacting the immature dendritic cells with a dendritic cell maturation factor. The dendritic cell maturation factor, may actually be one or more different substances, and may be provided by substances including, but not limited to PBMC conditioned media, maturation factors purified from the conditioned medium, and SACS (fixed *Staphylococcus aureus* Cowan 1 strain (Pansorbin)). Culture of the pluripotential cells with cytokines such as for example, a combination of GM-CSF and IL-4 or IL-13 stimulates the differentiation of the pluripotential cells to immature dendritic cells.

**[0013]** The method provided by this invention is particularly useful with human cells and provides sufficient numbers of mature dendritic cells to be useful for priming with antigen in vitro and activating an individual's T cells by administering the primed dendritic cells to the individual, or activation of T cells in vitro which are then administered to the individual. Accordingly, another aspect of this invention is a method of activating an individual's T cells comprising:

- a) obtaining a population of pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics from an individual and placing them in culture;
- b) contacting the pluripotential cells of step a with at least one cytokine to produce immature dendritic cells;
- c) contacting the immature dendritic cells with a dendritic cell maturation factor for a time sufficient to cause said immature dendritic cells to stably express dendritic cell characteristics;
- d) contacting the dendritic cells obtained from step c with an antigen to produce primed dendritic cells;
- e) exposing T cells to the primed dendritic cells of step d to activate said T cells. It is to be understood that one or more of the above steps may be combined so as to occur concurrently, such as for example, steps c and d.

**[0014]** Activation of T cell responses against a wide variety of antigens is facilitated by this invention. Such antigens include, but are not limited to viral, bacterial, tumor and self antigens such as antigen receptors, e.g. T cell receptors. Accordingly, the dendritic cells prepared according to this invention are useful for the prevention and treatment of various diseases including infectious disease, cancer and autoimmune disease.

**[0015]** The methods of promoting maturation of dendritic cells to mature dendritic cells using cytokines and a dendritic cell maturation factor, and the subsequent activation of T cells, may also be applied to individuals in vivo. According to this embodiment, an individual may be administered cytokines, e.g. GM-CSF and IL-4, or G-CSF to stimulate in vivo the production of immature dendritic cells. A dendritic cell maturation factor, such as conditioned medium, or a substance which stimulates the release of a dendritic cell maturation factor from PBMCs, may also be administered to an individual to stimulate in vivo maturation of immature dendritic cells, the population of which may optionally have been increased through prior administration of cytokines.

**[0016]** This invention also encompasses the mature dendritic cells prepared according to the method of the invention

and pharmaceutical compositions comprising the mature dendritic cells and a physiological carrier. Such carriers may include the media described below.

[0017] Another aspect of this invention is a culture medium useful for causing the maturation of immature dendritic cells to phenotypically stable, mature dendritic cells. The culture medium of the invention comprises a mixture of salts, carbohydrates and vitamins at physiologic concentrations typical of commercially available culture medium; as well as about 1-5%, and more preferably 1% human serum or plasma; GM-CSF and one or more cytokines which promote maturation of the PBMC phenpotential cells to immature dendritic cells and in amounts together sufficient to promote maturation of pluripotential PBMC to immature dendritic cells; and a sufficient concentration of a dendritic cell maturation factor to cause the maturation of immature dendritic cells to stable mature dendritic cells. This method can also be used with serum-free medium such as for example, XVIVO-20 or AIM-V.

[0018] Another aspect of this invention is an assay to detect a dendritic cell maturation factor. Such an assay is useful for the identification and production of the factor present in PBMC conditioned medium which causes the maturation of immature dendritic cells to the mature dendritic cells provided by this invention. This assay comprises contacting a test sample with a culture of unstable, immature dendritic cells derived from a population of pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics and which express characteristics of immature dendritic cells when cultured in a medium containing at least one cytokine. The presence of a dendritic cell maturation factor in the test substance is then determined by detecting the maturation of the immature dendritic cells in response to the test substance.

[0019] Another embodiment of the invention are combinations of cytokines which may be used in place of conditioned medium as the dendritic cell maturation factor.

[0020] It is an object of this invention to provide methods and compositions useful for activating an individual's T cells against specific antigens. The activation of an individual's T cell is useful for the prevention or treatment of disease, for example killer cells to treat or vaccinate against cancer or infection.

[0021] It is another object of this invention to provide methods useful for causing the stable maturation of immature dendritic cells to mature dendritic cells suitable for administering to an individual for the purpose of activating their immune response, and in particular, T cells.

[0022] Another object of this invention is to provide mature dendritic cells cultured in vitro from non-proliferating pluripotential cells.

[0023] Another object of this invention is to provide culture mediums which promote the maturation of immature dendritic cells to stable mature dendritic cells.

[0024] Yet another object of this invention is to provide an assay useful for detecting the presence in a test sample of a dendritic cell maturation factor.

[0025] Another object of this invention to provide methods for stimulating the maturation of dendritic cells in vivo.

## BRIEF DESCRIPTION OF THE FIGURES

### [0026]

**FIGURE 1:** Conditioned medium (CM) is required to ensure the maturation of dendritic cells from progenitor cells. ER- cells were cultured for 7 days in RPMI medium containing 1% plasma in the presence of GM-CSF and IL-4. Cells were then transferred to fresh plates and cultured for 4 days in the presence [open squares] or absence [closed circles] of CM. Control cultures were ER- cells cultured in Teflon beakers for 11 days [open circles]. T cell stimulatory function [primary allogeneic MLR] of the various APC populations is shown.

**FIGURE 2:** Cytofluorographic analysis of dendritic cells grown in the presence of GM-CSF/IL-4 and CM. Dead cells and contaminating lymphocytes were excluded by light scatter properties. Dot plots of the remaining cells are shown. Isotype controls are shown in the left panel.

**FIGURE 3:** Morphology and phenotype of blood derived dendritic cells and macrophages. Dendritic cells [left panel] were generated from normal blood ER- cells in the presence of GM-CSF/IL-4 and CM, cytospun onto glass slides and stained with a panel of mAbs [see Methods]. Macrophages were syngeneic ER- cells, enriched by plastic adherence and cultured for two weeks in Teflon beakers. CD8 serves as the isotype control antibody. Dendritic cells are distinguishable from macrophages by their dendritic shapes, lack of CD14 expression, high p55 expression and perinuclear pattern of CD68 expression. Black arrows in the right panel point to presumptive dendritic cells contaminating the macrophage populations.

**FIGURE 4:** Attempts to replace conditioned medium with cytokines. ER- cells were cultured in GM-CSF/IL-4 for 7 days and then transferred to fresh tissue culture plates. The cells were supplemented with 50% CM [black circles]

in the absence or presence of polyclonal neutralizing antibodies to  $\text{TNF}\alpha$ , IL-1 $\beta$  and IL-6 [1-5  $\mu\text{g/ml}$ ]. Cocktail refers to the combination of the various antibodies. Some cultures received TNF alpha [20 ng/ml] or IL-1 beta [10ng/ml] instead of CM. After 4 days [day 11 of culture], the cells were evaluated for T cell stimulatory activity in an allo MLR. Results are shown as  $^3\text{H-TdR}$  incorporation [cpm  $\times 10^{-3}$ ] and are averages of triplicates.

**FIGURE 5:** Dendritic cell progenitors are enriched in the CD14 hi population. ER- cells from two different sources were sorted into CD14 hi and lo populations as described in Methods. Unsorted and various sorted fractions were analyzed for T cell stimulatory activity in an MLR. In [A], the CD14 hi cells were irradiated with 3,000 rads, 137 Cs source. In [B], the CD14 hi cells were irradiated with 1,500 rads. Results are shown as  $^3\text{HTdR}$  incorporation [cpm  $\times 10^{-3}$ ] and are averages of triplicates.

**FIGURE 6:** Morphological stability of mature dendritic cells. Lymphocyte-depleted PBMC were cultured for 10 days in FCS-containing RPMI medium in the presence of GM-CSF and IL-4. Phase contrast micrographs were taken to illustrate the morphology of the cells. [A] No CM was added during the 10 day culture. Cells were washed on day 10 and recultured without cytokines for 3 additional days. They re-adhere firmly and have the appearance of macrophages. [B] CM was added from day 7-10. The cells acquire many lamellipodia or veils and are nonadherent. [C,D] CM was added from day 7-10, the cells were washed and returned to culture for 3 days without CM or cytokines. The morphology of mature dendritic cells persists both in RPMI/10% FCS [C] and X-VIVO 20/serum-free [D] media. A, x275; B-D, x550.

**FIGURE 7:** Comparative cytofluorographic analysis of mature vs. immature dendritic cells. Lymphocyte-depleted PBMC were cultured for 7 days with GM-CSF and IL-4 followed by a maturation period from day 8 to day 10 in the presence of CM. The resulting fully mature dendritic cells were compared to corresponding immature dendritic cells that had not been treated with CM. In all cases mature dendritic cells express CD83 and enhanced levels of CD86, and they lose CD115 expression. [A,A'] Dendritic cells cultured from day 7 to day 10 without [A] or with [A'] CM in RPMI/FCS are shown. Fluorescence on the x-axis represents HLA-DR expression; y-axis shows the indicated antibody staining. Dot plots are from cells gated on the basis of their light scatter properties [FSC/SSC]. [B,B'] Cells were analyzed on day 7[B] and on day 10, i.e., after maturation in the presence of CM [B']. Culture medium was RPMI/1% human plasma; lymphocytes were depleted with immunomagnetic beads. Histograms are from cells gated on the basis of their light scatter properties [FCS/SSC]. [C,C' - left] Like B,B', but lymphocytes were initially depleted by E-rosetting. [C,C' - middle] like B,B', but AIM-V medium/1% human plasma was used. [C,C' - right] like A,A', co-2b, isotype-matched control IgG2b [for CD83, 86, 14]; co-rat, control for rat IgG1 [for CD115]; FSC/SSC, forward scatter/side scatter. All markers are set according to staining with isotype-matched control antibodies.

**FIGURE 8:** Fixed staphylococci [SACS] are required to ensure the maturation of dendritic cells from progenitor cells. Lymphocyte-depleted PBMC were cultured for 7 days in RPMI medium supplemented with FCS and GM-CSF/IL-4. Cells were then transferred to fresh plates and cultured for 3 more days in the presence [closed squares] or absence [open squares] of SACS. T cell stimulatory function in the primary allogeneic MLR of the dendritic cells matured in SACS containing medium is markedly enhanced. Neither IL-1 $\beta$  [in A] nor IL-15 [in B] that were given instead of SACS could fully reconstitute the effect.

**FIGURE 9:** Morphology and motility of mature dendritic cells grown in the presence of GM-CSF/IL-4 and CM. Dendritic cell progenitors were primed for 7 days in the presence of GM-CSF and IL-4 followed by a 3 day maturation period in the presence of CM. Culture medium was RPMI supplemented with 1% autologous human plasma. Note the numerous thin cytoplasmic processes ("veils"). Photographs of the same field taken at intervals of 15 seconds show that the veils move. Magnification: x550.

**FIGURE 10:** Phagocytosis of latex beads. Dendritic cells were grown in the presence of GM-CSF and IL-4 until day 8. GCS-containing RPMI medium was used. Cells in the upper panel were exposed to latex beads for 24 hours in the absence of CM. Latex is taken up into the cells (arrows). In the lower panel cells were first allowed to mature in the presence of CM from day 7 to day 10 and were then exposed to latex. Little or no latex is phagocytosed into mature, "veiled" cells. Only a contaminating adherent cell has accumulated beads. x300.

**FIGURE 11:** Immunostimulatory functions of mature dendritic cells. Dendritic cells were cultured in the presence of GM-CSF and IL-4 until day 7 and further on until day 10 with [closed symbols] or without [open symbols] CM. RPMI was supplemented with FCS in A and D-F, and with 1% autologous human plasma in B and C. [A] Note pronounced enhancement of T cell stimulatory capacity in the MLR following exposure to CM. Two independent experiments are shown. [B] Fully mature dendritic cells grown in RPMI supplemented with 10% FCS or 1% autologous human

plasma were compared to each other. [C] Fully mature dendritic cells efficiently stimulate naive cord blood T cells. [D] Tetanus toxoid protein [1 µg/ml] is presented to a peptide-specific T cell clone. CM-induced maturation leads to a decrease in antigen processing capacity. Triangles in D indicate the [lack of] response of clone cells to APC in the absence of antigen. Values are means of triplicate wells and are expressed as cpm x 10<sup>-3</sup>. [E,F] Peptide-pulsed mature dendritic cells elicit T cell lines that lyse autologous target cells in a peptide dose-dependent [E] and MHC-restricted [F] manner. anti-cl. II, anti-cl. I, anti-MHC class II and I mAb's W6/32 and L243, respectively.

**FIGURE 12:** Dendritic cell progenitors reside in a CD34-negative population. PBMC of a cancer patient who had been treated with G-CSF were passed through an anti-CD34 immunoaffinity column. The original unseparated population [solid lines] and the CD34-depleted population [dashed lines] were cultured in RPMI\FCS with GM-CSF and IL-4 until d7 and further on from d7 to d10 in the presence [closed symbols] or absence [open symbols] of CM. Both populations gave rise to dendritic cells that matured upon exposure to CM.

**Figure 13:** Contribution of TNFα to the maturation of dendritic cells. T cell depleted blood mononuclear cells were cultured for 7 days in GM-CSF/IL-4 as described in Methods. From day 7-11 they were cultured in conditioned medium [MCM] or no additional supplementation [- MCM]. TNFα [50 ng/ml] was added every other day from days 0-8. On day 11, the cells were analyzed for CD83 expression. Residual cells were washed twice and returned to culture for three more days in RPMI containing 1% plasma. On day 14 the cells were tested for T cell stimulatory capacity in the allo MLR [left panel] and reevaluated for CD83 expression. The recovered cell yields from a starting volume of 40 ml of blood are given in the upper right hand corner of each FACS panel. The results are representative of two experiments.

**Figure 14:** MCM is more effective than a combination of cytokines in inducing dendritic cell maturation. T cell depleted blood mononuclear cells [ER- cells] were cultured for 7 days in GM-CSF/IL-4. They were washed twice, transferred to fresh six well plates and cultured in MCM, a combination of cytokines, or no additional supplementation [- CM]. On day 11 of culture [A and B], the cells were evaluated for T cell stimulatory activity in an allogenic MLR. In C and D, dendritic cells were washed twice and returned to culture for 1 [C] or 3 [D] more days in RPMI supplemented with 1% autologous human plasma. A and B, cytokines were IL-1 [20 ng/ml], IL-6 [20 ng/ml], IFNα [.02 ng/ml] and TNFα [20 ng/ml]. In C and D, the cocktail of cytokines mimicked the concentrations measured by ELISA in the corresponding MCM. C: IL-1 [92 ng/ml], IL-6 [1 µg/ml], IFNα [.02 ng/ml] and TNFα [182 ng/ml]; D: IL-1 [20 ng/ml], IL-6 [734 ng/ml], IFNα [.08 ng/ml] and TNFα [24 ng/ml]. The values represent the averages of triplicates +/- S.D.

**Figure 15:** Maturation of dendritic cells induced by MCM is irreversible. GM-CSF/IL-4 treated ER- cells were cultured for 4 days in medium alone [-MCM], two different MCM preparations [MCM A and B], or a combination of cytokines equal to that present in the MCM. On day 11, the cells were washed and analyzed for CD83 expression. Residual cells were returned to culture for 3 more days [day 14] in the absence of further supplementation and analyzed for the retention of CD83. MCM contained the following cytokines: A; IL-1 [92 ng/ml], IL-6 [1 µg/ml], IFNα [.02 ng/ml] and TNFα [182 ng/ml]; B; IL-1 [20 ng/ml], IL-6 [734 ng/ml], IFNα [.08 ng/ml] and TNFα [24 ng/ml].

**Figure 16:** Treatment with MCM is superior to cytokines ER- cells were cultured for 7 days in GM-CSF and IL-4, and recultured in the presence of MCM, cytokine combinations or no additional supplementation [-MCM] for 4 more days. On day 11, cells were collected and counted. The percent yields reflect the number of cells obtained relative to treatment with MCM [100%]. The cells were then washed to remove residual cytokines, cultured for 3 days in RPMI with 1% plasma in the absence of additional supplements and analyzed for CD83 expression [day 14]. Values are the mean +/- SD and are representative of 5-7 experiments. \*p<0.05, paired student's t test.

#### DETAILED DESCRIPTION OF THE INVENTION

**[0027]** This invention relates to methods and compositions useful for promoting the maturation of immature dendritic cells to a mature and stable phenotype as well as to the stable mature dendritic cells. The mature dendritic cells are useful as antigen presenting cells (APCs) which activate other immune cells including antigen specific helper and killer T cells. Thus, this invention also provides methods and compositions useful for the treatment of disease.

**[0028]** The method of producing mature dendritic cells according to this invention comprises contacting immature dendritic cells with a dendritic cell maturation factor. We have found such a dendritic cell maturation factor in conditioned medium obtained from cultures of peripheral blood mononuclear cells (PBMCs). Immature dendritic cells may be derived from PBMCs by culturing PBMCs with cytokines, such as, for example, a combination of GM-CSF and IL-4, which promote their differentiation to immature dendritic cells. Surprisingly, we have determined that unless they are

exposed to a dendritic cell maturation factor, removal of the cytokines from contact with the cells causes the cells to revert back to a pluripotential cell having characteristics similar to macrophages.

**[0029]** The response of immature dendritic cells to contact with the maturation factor is an increased expression of CD83 and p55 dendritic cell markers; strong expression of antigen presenting MHC class I and II products; and expression of several accessory (adhesion and co-stimulatory) molecules including CD40, CD54, CD58, CD80 and CD86. A decrease in expression of CD115 as well as CD14, CD68 and CD32, which are markers associated with immature dendritic cells, is also observed as a result of carrying out the method of this invention. In addition, this phenotype remains stable for up to three days even after removal of cytokines used to promote cell maturation.

**[0030]** We have developed an in vitro culture system whereby dendritic cells are induced to undergo irreversible maturation. The method utilizes a CD34<sup>-</sup>, CD14<sup>+</sup> enriched precursor population in blood and entails two stages. The first is a priming phase by GM-CSF and IL-4 that yields cells which actively internalize particulate antigens, and which are highly efficient at priming resting T cells to native soluble antigens (Bender, 1996; Romani, 1996; Sallusto, 1994). Phenotypically, these "immature" dendritic cells are characterized by moderate levels of MHC and costimulator molecules, and variable levels of the macrophage associated molecules CD14 and CD32 [FcR] (Bender, 1996; Romani, 1996; Sallusto, 1994).

**[0031]** The second or maturation step in dendritic cell development requires exposure to MCM (or components thereof). We define maturation as the irreversible acquisition of several properties: typical stellate morphology; non-adherence to plastic; upregulation of MHC and co-stimulator molecules; and expression of the two dendritic cell restricted molecules CD83 (Zhou, 1995) and p55 (Mosialos, 1996). Maturation is typically marked by a coordinate series of additional changes that include downregulation of macropinocytosis, expression of activation molecules [CD25, CD95, CD45RO] and perinuclear CD68, and the loss of CD1a, CD32, CD115 and CD14 (Bender, 1996; Romani, 1996). The mature dendritic cells are less efficient at processing soluble antigens but highly efficient at presenting peptide antigens to T cells.

#### Sources of Pluripotential Cells

**[0032]** The pluripotential cells, from which the immature dendritic cells for use in this invention are derived, are present in blood as PBMCs. Although most easily obtainable from blood, the pluripotential cells may also be obtained from any tissue in which they reside, including bone marrow and spleen tissue. These pluripotential cells typically express CD14, CD32, CD68 and CD115 monocyte markers with little or no expression of CD83, p55 or accessory molecules such as CD40 and CD86. When cultured in the presence of cytokines such as a combination of GM-CSF and IL-4 or IL-13 as described below, the pluripotential cells give rise to the immature dendritic cells.

**[0033]** Methods of obtaining PBMCs from blood, such as differential sedimentation through an appropriate medium, e.g. Ficoll-Hypaque [Pharmacia Biotech, Uppsala, Sweden], are well known and suitable for use in this invention. In a preferred embodiment of the invention, the pluripotential cells are obtained by depleting populations of PBMCs of platelets, and T and B lymphocytes. Various methods may be used to accomplish the depletion of the non-pluripotential cells. According to one method, immunomagnetic beads labelled with antibodies specific for T or B lymphocytes, either directly or indirectly may be used to remove the T and B cells from the PBMC population. T cells may also be depleted from the PBMC population by rosetting with neuraminidase treated red blood cells as described by O'Dherthy (1993), which is incorporated herein by reference.

**[0034]** To produce 3 million mature dendritic cells, it is necessary to process about 40 mls of blood. 4 to 8 x 10<sup>7</sup> pluripotential PBMC give rise to approximately 3 million mature dendritic cells.

#### Culture of Pluripotential PBMCs To Produce Immature Dendritic Cells

**[0035]** The immature dendritic cells for use in this invention are post-mitotic but not yet terminally differentiated to either a macrophage or dendritic cell phenotype. Cultures of immature dendritic cells may be obtained by culturing the pluripotential cells in the presence of cytokines which promote their differentiation. A combination of GM-CSF and IL-4 at a concentration of each at between about 200 to about 2000 U/ml, more preferably between about 500 and 1000 U/ml, and most preferably about 800 U/ml (GM-CSF) and 1000 U/ml (IL-4) produces significant quantities of the immature dendritic cells. A combination of GM-CSF (10 ng/ml) and IL-4 (10-20 ng/ml) has been found to be useful with this invention. It may also be desirable to vary the concentration of cytokines at different stages of the culture such that freshly cultured cells are cultured in the presence of higher concentrations of IL-4 (1000 U/ml) than established cultures (500 U/ml IL-4 after 2 days in culture). Other cytokines such as IL-13 may be found to substitute for IL-4.

**[0036]** Methods for obtaining these immature dendritic cells from adherent blood mononuclear fractions are described in Romani et al. (1994); and Sallusto and Lanzavecchia, 1994) both of which are incorporated herein by reference. Briefly, lymphocyte depleted PBMCs are plated in tissue culture plates at a density of about 1 million cells/cm<sup>2</sup> in complete culture medium containing cytokines such as GM-CSF and IL-4 at concentrations of each at between about 800

to 1000 U/ml and IL-4 is present at about 1000 U/ml.

[0037] Various mediums are suitable to initially culture PBMCs. Typical media for use in this invention comprise physiologic inorganic salts, carbohydrates including sugars, amino acid, vitamins and other components known to those in the art. Preferred media formulations include, for example, RPMI 1640, X-VIVO 20, AIM-V, HybriCare and Iscove's. More preferably the medium is RPMI 1640 or X-VIVO 20 or AIM-V. Most preferably, the medium is RPMI 1640.

[0038] It is desirable to avoid the use of fetal calf serum (FCS) in cultures for human use. The presence of bovine proteins may cause unwanted sensitization of the recipient against bovine proteins and it is therefore desirable to substitute FCS with human serum or plasma in an amount sufficient to maintain cell viability. The use of autologous, non-heat-inactivated human plasma at a concentration of about 1% is preferred. Using RPMI 1640 supplemented with 1% autologous human plasma, a final yield of between about  $0.8$  to  $3.3 \times 10^6$  CD83+ mature dendritic cells is obtainable from 40 ml of blood.

[0039] Another source of immature dendritic cells are cultures of proliferating dendritic cell precursors prepared according to the method described in Steinman et al. International application PCT/US93/03141, which is incorporated herein by reference. Since the dendritic cells prepared from the CD34+ proliferating precursors mature to dendritic cells expressing mature characteristics it is likely that they also pass through a development stage where they are pluripotent. By treating these cultures with a dendritic cell maturation factor as described below, the efficiency of obtaining mature dendritic cells from proliferating precursors may also be improved.

#### Maturation of Immature Dendritic Cells

[0040] The mature dendritic cells are prepared according to this invention by contacting the immature dendritic cells with a dendritic cell maturation factor. As referred to herein, the dendritic cell maturation factor may actually be one or more specific substances which act in concert to cause the maturation of the immature dendritic cells. Such a factor has been determined to be present in PBMC conditioned medium, preferably monocyte conditioned medium. Another means of obtaining dendritic cell maturation factors are to treat PMBCs with substances which stimulate the release of such factors. Substances such as immunoglobulin or SACS (also referred to as Pansorbin) are useful for causing monocytes to release cytokines.

[0041] After the cells have been cultured for a sufficient time to express some characteristics of dendritic cells, preferably about 6 to 10 days, PBMC conditioned media is added to the culture medium at a final concentration of between about 10 to 50%. More preferably between about 25 and 50%. Maturation of the immature dendritic cells to the mature phenotype may occur without the presence in the medium of the cytokines used to stimulate maturation of the pluripotent cells to immature dendritic cells. Typically, the immature dendritic cells are obtained after about 6 to 7 days in culture with the cytokines. Preferably the immature dendritic cells are subcultured prior to addition of the dendritic cell maturation factor, e.g., conditioned medium.

[0042] In a preferred embodiment, the immature dendritic cells which are non-adherent cells are harvested and subcultured in the presence of the dendritic cell maturation factor present in the conditioned medium, and optionally with cytokines such as GM-CSF and IL-4. The mature dendritic cells are typically obtained within approximately three days following addition of the conditioned medium.

[0043] Variations in the culture protocol which are also considered within the scope of this invention may include, but not be limited to, changes in cytokines used to promote differentiation of PBMCs to immature dendritic cells, concentrations of cytokines and timing of the addition of the dendritic cell maturation factor present in the conditioned medium.

[0044] An alternative to causing stable maturation of dendritic cells using conditioned medium is the use of a composition comprising at least one cytokine but more preferably a combination of cytokines. Examples of cytokines which may be used alone or in combination with each other include  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6 and IFN $\alpha$ . The combination of cytokines as shown in Figure 16 are preferred for use in this invention to cause the stable maturation of dendritic cells. More preferably a combination comprises  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6, IFN $\alpha$ ;  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6;  $\text{TNF}\alpha$ , IL-1 $\beta$ , IFN $\alpha$ ;  $\text{TNF}\alpha$ , IFN $\alpha$ , IL-6; IL-1 $\beta$ , IL-6, IFN $\alpha$ ; and  $\text{TNF}\alpha$ , IL-1 $\beta$ . Most preferred is  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6, IFN $\alpha$ .

[0045] Concentrations of cytokines are used which optimize the number of cells which retain the stable mature dendritic cell phenotype. Such concentrations, including the ratio of concentrations, may be based on the concentration of the cytokines present in the conditioned medium described herein. The cytokine concentrations shown in Figures 14 and 15. More preferably the concentrations are: about 20 - 200 ng/ml  $\text{TNF}\alpha$ ; about 20 - 100 ng/ml IL-1 $\beta$ ; about 20 - 1000 ng/ml IL-6; and about 0.02 - 0.08 ng/ml IFN $\alpha$ . Most preferably the concentrations are: about 20 ng/ml  $\text{TNF}\alpha$ ; about 20 ng/ml IL-1 $\beta$ ; about 20 ng/ml IL-6; and about 0.02 ng/ml IFN $\alpha$ . These concentrations can be optimized by performing dose-response curves known to those skilled in the art. See, for example, Goodman and Gilman's The Pharmacological

Basis of Therapeutics, Eighth Ed.; Gilman et al. eds.; Pergamon Press.

Characterization of Conditioned Medium

**[0046]** The conditioned medium for use in this invention may be made by culturing PBMCs, preferably monocytes in basic growth medium. Preferably the cells for producing conditioned medium are cultured in the presence of a stimulus which stimulates the release of factors from the donor cells. An example of such a preferred factor is gamma globulin when bound to the culture substrate. Methods for producing PBMC (monocyte) conditioned medium have previously been described in O'Doherty et al. (1993 and 1994) which are incorporated herein by reference. PBMC or preferably T cell depleted, or more preferably T and B cell depleted are cultured (about  $5 \times 10^7$  cells/100 mm plate) for about 24 hours in medium (6-8 mls) containing 1% of plasma, preferably autologous plasma. Where conditioned medium is to be used for stimulating maturation of dendritic cells in vivo, the growth medium should be selected to be compatible with use in humans, e.g. X-VIVO or AIM-V and preferably is used without serum or plasma. For use in humans the conditioned medium is concentrated using standard procedures and adjusted to be isotonic so as to be suitable for administration in vivo. Using this method, equivalent concentrations of dendritic cell maturation factor can be achieved in vivo, as in vitro.

**[0047]** In addition to gamma globulin as a stimulant of cytokine release from the culture PBMCs, other cytokine stimulators such as SACS (fixed Staphylococcus Aureus Cowan 1 strain, 2,01 mg/ml Ig-binding capacity: Pansorbin cells, Cat. No. 507861) may also be used. Because SACS is bacterially derived its use is less desirable than Ig.

**[0048]** In addition to stimulating the production of cytokines for producing conditioned medium, SACS may also be substituted for conditioned medium as a dendritic cell maturation factor. When used to promote maturation of the immature dendritic cells, SACS is added to cultures at a concentration sufficient to cause their maturation, preferably at about 1:10000 dilution.

Response of Immature Dendritic Cells to Dendritic Cell Maturation Factors

**[0049]** This invention provides evidence for a new pathway for dendritic cell development. Prior studies have indicated that dendritic cells arise either from CD34+ proliferating progenitors (Inaba et al. 1992; Caux et al. 1992), or from non-proliferating "null" cells that lack monocyte and lymphocyte markers [CD3, CD14, CD16, CD19] (O'Doherty et al. 1993). Both of these progenitor populations are infrequent in human blood representing <0.1% and <1% of blood mononuclear cells, respectively. Sallusto and Lanzavecchia [personal communication] reason that monocytes can give rise to dendritic cells in the presence of FCS, but we find that these cells are not fully mature in several respects. In contrast, large numbers of mature dendritic cells can develop, and in human plasma or serum, when CM is added to the GM-CSF and IL-4 priming step. The precursors are radioresistant and primarily in the standard "monocyte" fraction [CD14 positive and plastic adherent]. It is possible that most monocytes are bipotential and can develop into either macrophages or dendritic cells depending upon the cytokines that are applied. Alternatively, only some of the "monocyte" fraction may have the potential to develop into dendritic cells. At this time, our data are consistent with the latter possibility since the total yield of dendritic cells from radioresistant precursors represents about 5.5-16% of the starting number of monocytes.

**[0050]** Physiologic counterparts for the maturation over several days of dendritic cells from nonproliferating blood progenitors can be proposed in at least three settings. First sizable numbers of dendritic cells traffic in afferent lymphatics, and these cells derive from precursors that have been proliferating 3-7 days prior to detection in lymph (Mason et al. 1981; Pugh et al. 1983; Fossum, 1989a; Fossum, 1989b). These afferent lymph dendritic cells may derive from blood progenitor cells that undergo a priming phase. Second, dendritic cells are rapidly induced to migrate into tissues [lung, gut] in response to pro-inflammatory stimuli e.g. LPS or infection (Watson et al. 1990; McWilliam et al. 1994). There they may undergo further maturation in response to cytokines that are produced locally by tissue macrophages.

**[0051]** Third, significant numbers of mature dendritic cells accumulate in rheumatoid synovial exudates during acute flares of arthritis (Zvaifler et al. 1985; Bhardwaj et al. 1988; Helfgott et al. 1988; Thomas et al. 1994). These effusions are rich in GM-CSF, IL-4, IL-6 and TNF alpha, amongst other cytokines [reviewed in (Klareskog et al. 1995)]. In addition, the presence of substantial numbers of macrophages and immune complexes may provide a natural "conditioned medium" for the maturation and differentiation of dendritic cells from blood progenitors that enter inflamed joints.

**[0052]** Based on the data reported in Examples 1 and 2, dendritic cells cultured from peripheral blood precursors in GM-CSF and IL-4 for 6-7 days can be considered as immature dendritic cells. Although they have already acquired relatively high levels of MHC and adhesion/costimulator molecules as well as the capacity to stimulate resting T cells they still lack markers for terminal dendritic cell differentiation such as CD83. They are however well equipped with the necessary prerequisites for the processing of native protein antigens. They can phagocytose particulate matter and they can efficiently process native tetanus toxoid protein into immunogenic MHC-peptide complexes recognized by a tetanus peptide-specific T clone. In addition, Sallusto et al. (1995) described active uptake of soluble macromolecules by macropinocytosis, and we find uptake of latex particles as well. Immature dendritic cells may therefore be useful in immunization protocols that use native proteins or particles as antigens. One must keep in mind, however, that the immature

dendritic cells generated with GM-CSF and IL-4, but without CM, are not stable. Upon withdrawal of cytokines they re-adhere and appear to revert back to monocytes. Such cells would therefore not be as useful for therapeutic approaches. It will be preferred to induce maturation of antigen-pulsed dendritic cells by the method described here, i.e., exposure to monocyte-conditioned media.

5 [0053] Dendritic cells grown in GM-CSF and IL-4 for 7 days and exposed to macrophage-conditioned media for another three days develop into mature dendritic cells. These cells have down-regulated their antigen uptake mechanisms and their processing capacity. CM- treated dendritic cells correspond morphologically, phenotypically and functionally to well defined populations of mature dendritic cells, e.g., cultured epidermal Langerhans cells (Romani et al. 1989b); Tenunissen et al. 1990), cutaneous dendritic cells obtained by emigration from skin explants (Pope et al. 1995; 10 Lenz et al. 1993), or blood dendritic cells obtained by classical methods involving 36 hours of culture (Young and Steinman, 1988). They are specialized in the sensitization of naive, resting T cells. We show here that they can efficiently induce proliferation in allogeneic umbilical cord T cells which may be considered virgin T cells. We also demonstrate that these mature dendritic cells can be loaded with an antigenic peptide and induce antigen-specific cytotoxic T lymphocytes from populations of autologous PBMC or CD8+ T cells. In addition, mature dendritic cells in small numbers 15 are able to elicit rapid and virus-specific CTL responses from quiescent autologous T cells. Mature dendritic cells will be helpful in immunization protocols that employ antigenic peptides preferably to proteins. This applies especially to the field of tumor immunotherapy. Tumor-specific peptides are being discovered at a fast rate (Boon et al. 1994).

[0054] Based on our data, the optimal method to generate mature dendritic cells for clinical purposes would be to deplete PBMC of lymphocytes with immunomagnetic beads, culture them for 6 to 7 days in RPMI medium supplemented with 1% autologous human plasma and GM-CSF/IL-4, and induce them to mature within another three days of 20 culture by the addition of conditioned medium that is produced by PBMC adhering to Ig-coated Petri dishes. All these reagents are already approved [Anti-mouse Ig Dynabeads and mouse anti-T and B cell mAb's/Baxter; GM-CSF/Sandoz; IL-4/Schering-Plough; Ig/Biochemie-Sandoz] or have been used in clinical studies [RPMI medium, Lymphoprep].

[0055] Our method to generate dendritic cells from progenitor cells in autologous plasma will be useful in several 25 respects. First, the method is simple, reproducible and generates  $1-3 \times 10^6$  dendritic cells from relatively small, 40-50 ml blood samples. Second, critical features of dendritic cells such as pathways of antigen presentation and T cell signalling can be studied in the absence of foreign FCS derived proteins. Third, one has ready access to cells that can be used as adjuvants to enhance protective immune responses *in vivo*. We find that dendritic cells generated by the method described here and pulsed with either influenza virus [live or heat inactivated] or immunodominant peptides 30 [Table 6] induce virus-specific CD8+ killer cell responses *in vitro*.

#### Pulsing Dendritic Cells With Antigen

[0056] The mature dendritic cells prepared according to this invention are useful for activating T cells against specific 35 antigens. Several antigens and methods for priming dendritic cells have been described and may be adapted for use in this invention. See, for example Engleman et al. International patent application PCT/US95/07461; Hsu et al., Nature Med. 2:52-58 (1996); and Steinman et al. International application PCT/US93/03141 which are all incorporated herein by reference. Antigens associated with fungal, bacterial, viral, tumor, or autoimmune (i.e., self antigens) diseases are useful for priming dendritic cells to activate T cells which aid in treating or preventing disease.

40 [0057] Where it is desirable for cells to take up antigen by phagocytosis, it is preferable to add antigen to the cultures of immature dendritic cells prior to addition of the dendritic cell maturation factor. Phagocytosis may be desirable when particulate antigens, or immune complexes are used. In most cases it is sufficient to expose antigen to the dendritic cells after they have attained the mature phenotype or while they are exposed to the dendritic cell maturation factor to attain the mature phenotype. This method is preferred when soluble peptide antigens are used.

45 [0058] For the purpose of priming cells typically approximately  $1$  to  $5 \times 10^6$  cells are exposed to antigen at a concentration of between about 10 pM to about 10  $\mu$ M, inclusive. More preferably about 1  $\mu$ M antigen to about 3 million cells is used. The dendritic cells are cultured in the presence of the antigen for a sufficient time to allow for uptake and presentation. Typically uptake and processing can occur within 24 hours but longer (up to and including 4 days) or shorter (about 1-2 hours) periods may also be used.

50 [0059] For activating T cells in an individual between about  $2 \times 10^5$  and  $2 \times 10^9$  more preferably between about 1 million and 10 million mature dendritic cells should be administered to the individual. The dendritic cells should be administered in a physiologically compatible carrier which is nontoxic to the cells and the individual. Such a carrier may be the cell growth medium described above. The mature dendritic cells prepared according to this invention are particularly potent at activating T cells. For example, using prior methods of obtaining dendritic cells the ratio of dendritic cells to T cells necessary for strong T cell activation is about 1 dendritic cell to 30 T cells whereas the ratio according to this invention 55 is about 1 to 1000. Thus, fewer dendritic cells are required. For activating T cells *in vitro*, the ratio of dendritic cells to T cells is between about 1:10 and 1:1000. More preferably between 1:30 and 1:150. Between approximately  $10^8$  and  $10^9$  activated T cells are administered back to the individual to produce a response against an antigen.

[0060] Dendritic cells may be administered to an individual using standard methods including intravenous, intraperitoneal, subcutaneously, intradermally or intramuscularly. The homing ability of the dendritic cells facilitates their ability to find T cells and cause their activation.

[0061] The methods of this invention are particularly well suited for use against tumors from which tumor specific antigens are obtainable or which express a mutated protein. Preferably, the antigen is a molecule required by the tumor cells to be malignant, for example altered ras. Non-limiting examples of tumors for which tumor specific antigens have been identified include melanoma, B cell lymphoma, uterine or cervical cancer. An example of a melanoma antigen which could be considered for use to prime dendritic cells is the human melanoma specific antigen gp75 antigen (Vijayashanti S. et al., *J. Exp. Med.*, 171:1375-1380 (1990)). An example of an antigen useful for targeting cells against cervical cancer is papilloma virus. Tumor specific idiotype protein derived from B cell lymphomas has been reported to prime dendritic cells which activate T cells against the tumor cells. Hsu et al. (1966), supra. In addition to tumor antigens, viruses may also be used to prime the dendritic cells. Viruses may also be used to cause dendritic cells to present viral antigens, or they may be engineered to express non-viral proteins which are then processed and presented by the dendritic cells. Non-limiting examples of viruses which may be used to prime dendritic cells include, but are not limited to, influenza, HIV, CMV, EBV, human papilloma virus, adenovirus, HBV, HCV and vaccinia. Isolated viral proteins, inactivated or attenuated virus may also be used. Non-limiting examples of bacterial or protozoan antigens include tetanus toxoid, BCG, malaria antigens and leishmania antigens. Specific antigens associated with autoimmune disease such as immune receptors may also be used to prime dendritic cells to be used to activate T cells against such antigens.

#### Method of Assaying for a Dendritic Cell Maturation Factor

[0062] This invention provides an assay useful for identifying the dendritic cell maturation factor present in a test substance such as monocyte conditioned medium. This assay is based on the ability of immature dendritic cells to mature and express a stable phenotype after having been contacted with a dendritic cell maturation factor. Thus, the assay comprises contacting a culture of immature dendritic cells with a test substance and detecting the maturation of the immature dendritic cells in response to the presence of the test substance. The immature dendritic cells for use in this assay are derived from a population of pluripotential cells, such as human PBMCs having the potential of expressing either macrophage or dendritic cell characteristics and which express characteristics of immature dendritic cells when cultured in a medium containing cytokines (e.g., GM-CSF and IL-4). Maturation associated with the presence of a dendritic cell maturation factor may be confirmed, for example, by detecting either one or more of 1) an increase expression of one or more of p55, CD83, CD40 or CD86 antigens; 2) loss of CD115, CD14, CD32 or CD68 antigen; or 3) reversion to a macrophage phenotype characterized by increased adhesion and loss of veils following the removal of cytokines which promote maturation of PBMCs to the immature dendritic cells. The assay described above may be used in conjunction with biochemical techniques to identify the dendritic maturation factor present in monocyte CM. In addition, it provides a useful bioassay to confirm the activity of such a factor once it is identified or purified in connection with its manufacture.

[0063] In summary, mature human dendritic cells can be generated in substantial numbers from nonproliferating progenitors in human blood using a two step protocol. T cell depleted mononuclear cells are first cultured with, for example, GM-CSF and IL-4, and then exposed to dendritic cell maturation factor, preferably MCM. The dendritic cells generated using this approach are rendered terminally mature and are the most potent antigen presenting cells identified to date in humans. MCM contained substantial, although varying quantities of several factors including  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$ , and  $\text{IFN}\alpha$ . These four factors, individually or in various combinations can substitute for the MCM to generate irreversibly differentiated dendritic cells. However, the yields, percentage of cells expressing the mature phase marker CD83, and MLR stimulatory function were lower when defined cytokines were used in the place of MCM. Therefore, the full maturation of dendritic cells, because it entails changes in many known cell and molecular properties, requires a number of different cytokines that are released in tandem from appropriately stimulated monocytes. MCM is the most potent factor for generating irreversibly differentiated dendritic cells, however, the use of defined cytokines may be a more advantageous and convenient method for treatment in a clinical setting. Therefore, this invention provides a method of using defined cytokines which provide nearly equal activity for generating irreversibly differentiated dendritic cells to the use of MCM.

[0064] **Abbreviations used:** APC, antigen presenting cells; CTL, cytolytic T cell responses; ER+, T cell enriched; ER-, T cell depleted; MCM, monocyte conditioned medium; MLR, mixed leukocyte reaction; PBMC, peripheral blood mononuclear cells.

EXAMPLESEXAMPLE 15 MethodsCulture medium

10 **[0065]** RPMI 1640 supplemented with 20 µg/ml of gentamicin, 10 mM HEPES, and either 10% heat-inactivated FCS [Gemini Bioproducts, Calabasas, Ca], 1% to 10% autologous plasma [heparinized] or 5% single donor human serum.

Cytokines

15 **[0066]** We were generously supplied with rhGM-CSF [S.A.  $1 \times 10^5$  U/ug] by Kirin Brewery Co., Maebashi, Gunma, Japan, and rhIL-4 by Immunex Corp. Seattle, WA [S.A.  $0.5 \times 10^5$  U/ug] and Schering-Plough Corp. Union, NJ [S.A.  $2.865 \times 10^7$  U/mg]. The following cytokines were purchased: TNFα [Endogen, Cambridge, Ma], IL-1β and IL-6 [R and D Corp, Minneapolis, Mn], IFNα [Pestka Biomedical Labs, Inc. West Caldwell, NJ]. Elisa kits were obtained from Pestka Biomedical Labs, Inc. [IFNα] and R and D Corp [TNF α, IL-1β, IL-6].

20 Generation of dendritic cells from human blood

**[0067]** Peripheral blood was obtained from normal donors in heparinized syringes, and PBMC isolated by sedimentation in Ficoll-Hypaque [Pharmacia Biotech, Uppsala, Sweden]. T cell-enriched [ER+] and T cell-depleted [ER-] populations were prepared by rosetting with neuraminidase treated-sheep red blood cells as described (Carr and Kim, 25 1993). Different starting populations of PBMC were plated in 3 ml volumes in 6 well tissue culture dishes [Falcon] in complete medium. They consisted of [a] plastic adherent and nonadherent PBMC [obtained after 60 min adherence of  $6 \times 10^6$  PBMC/well]; [b] ER- cells; [c] ER-cells sorted on a FACStar<sup>Plus</sup> into CD14 high or low cells. Groups [b] - [c] were plated at  $2 \times 10^6$ /well. GM-CSF and IL-4 were added at final concentrations of 1000 U/ml on the initial day of culture. Cytokines were replenished every other day [days 2, 4 and 6] by removing 0.3 ml of the medium and adding back 0.5 30 ml fresh medium with cytokines. On day 7, non-adherent cells were collected by moderately vigorous aspiration and analyzed immediately, maintained in culture or transferred to new 6 well plates. When cultured beyond 7 days, the cultures were supplemented with Pansorbin, [1:10,000 dilution of 2 mg IgG/ml, Calbiochem, La Jolla, Ca], or conditioned medium [final concentration 33 or 50% v/v, see below] at day 7. In some experiments, the cultures were supplemented with monocyte conditioned medium [MCM, final concentration 50% v/v] or various combinations of cytokines and harvested on days 10-11. In some experiments, cells were washed out of supplemental cytokines or MCM at day 7 or day 35 11 before use in phenotypic or functional assays.

T cells

40 **[0068]** T cells were purified from ER+ cells by removal of monocytes, NK cells, and MHC class II+ cells as described (Bender, 1995; Bhardwaj, 1994).

Conditioned medium

45 **[0069]** Ig coated bacteriologic plates [100 mm, Falcon, Lincoln Park, N.J.] were prepared immediately prior to use by the addition of 4 ml of human gamma-globulin [10 mg/ml, Cappel Labs, Organon Teknika, Westchester, Pa] for 1 min. The residual gamma-globulin was removed and saved for reuse at least 4-6 times. The plates were washed three times with phosphate buffered saline [PD] prior to use. T cell-depleted ER- cells [ $5 \times 10^7$ ] were layered onto the Ig-coated bacteriologic plates for 1 hour in volumes of 6-8 ml. Non-adherent cells were washed off with gentle aspiration. The 50 gamma-globulin adherent cells were incubated in fresh complete medium with 1% autologous plasma at 37°C for 24 h. The medium was collected and frozen at -20°C prior to use. In some cases, conditioned medium was prepared from cells stimulated with pansorbin instead of human Ig.

Monoclonal Antibodies [mAbs]

55 **[0070]** MAbs towards the following antigens were used: HLA-DR, CD14, CD3, CD32, CD4/CD8, CD5, CD19, CD16, CD56, CD1a, CD54, CD58, CD25, CD45RO, CD11a, CD11b and CD11c, [Becton-Dickinson, Mountainview, CA], CD80 and CD86 [IgG1, FITC conjugate, Pharmingen], CD83, which is detected on mature blood dendritic cells [IgG1, PE con-

jugate, Coulter Corp, Miami, Florida], CD68 [Dako, Carpinteria, Ca], CD40, CD95/fas [Immunotech, Marseilles, France], p55 [K-2 clone, a gift from Dr. E. Langhoff; Mosialos et al. (1996)], mannose receptor [mAb 3.2PB1, a gift from Dr. A. Lanzavecchia], Lag anti-Birbeck granule associated antigen [a gift from Dr. S. Imamura], Ki 67 [MIB-1, IgG1, Dako]. Secondary antibody was PE-conjugated F[ab']<sub>2</sub> goat anti-mouse IgG [gamma and light chain, Tago, Burlingame, Ca].

#### Polyclonal neutralizing antibodies

[0071] Goat anti-human polyclonal antibodies were purchased from R and D Corp, Minneapolis, MN. They included antibodies toward TNF $\alpha$ , IL-1  $\beta$ , IL-1  $\alpha$ , IL-12 and IL-6. Control antibody was goat IgG [R and D Corp.].

#### Cytofluorography and cell sorting

[0072] Cell populations were phenotyped with the panel of Mabs listed above and analyzed on a FACScan. To obtain CD14<sup>+</sup> cells,  $2 \times 10^7$  ER- cells were stained with PE-CD14 [Becton-Dickinson, Mountainview, CA] for 30 min on ice, washed extensively and then sorted on a FACStar<sup>Plus</sup>. Both CD14 high and CD14 low cells were evaluated for the ability to develop into dendritic cells. Dead cells and contaminating lymphocytes were excluded by forward and side scatter properties.

#### Immunohistochemistry

[0073] Cytospins of various cell populations were prepared using a Cytospin 2 centrifuge [Shandon, Inc., Pittsburgh, PA]. Slides were fixed in absolute acetone for 5 min at room temperature and then incubated with mAbs for 45 min. Cytospins were then washed and incubated with 1:200 dilution of biotinylated goat anti-mouse IgG [Boehringer Mannheim Biochemicals, Indianapolis, IN] for 45 min, followed by a horseradish peroxidase [HRP]-biotin-avidin complex [ABC kit; Vector laboratories, Inc., Burlingame, CA] for 30 min. Non-bound HRP was then washed off, and the HRP reaction product was developed with H<sub>2</sub>O<sub>2</sub> and diaminobenzidine tetrahydrochloride [Polysciences, Warrington, PA].

#### Allogeneic MLR

[0074] To test for T cell stimulatory function, the APCs were unirradiated or irradiated [Gy 30] and added in graded doses as stimulators for  $2 \times 10^5$  purified, allogeneic T cells in 96 well flat bottomed plates [Costar]. Proliferation was determined on days 4-6 with the addition of 4  $\mu$ Ci/ml of [<sup>3</sup>H]TdR for 10-16 h to triplicate wells [mean cpm].

#### Induction of influenza virus specific CTL

[0075] Dendritic cells prepared from HLA A2.1<sup>+</sup> donors were washed out of medium containing serum and resuspended in RPMI to  $0.5 - 1 \times 10^7$  cells/ml. Live or heat-inactivated influenza virus [PR8, Spafas Inc., Storrs, Ct] was added at a final concentration of 1,000 HAU/ml for 1 hr at 37 $^{\circ}$  as previously described [Bhardwaj et al. 1994; Bender et al. 1995]. Following three washes,  $3 \times 10^4$  dendritic cells were added to purified T cells [ $1 \times 10^6$ ] in 48 well plates [Costar, Cambridge, Mass.] After 7 days, the T cells were assayed for cytolytic activity on uninfected or influenza-infected autologous macrophage targets using a conventional <sup>51</sup>Cr release assay [Bhardwaj et al. 1994]. Alternatively, targets were HLA A2.1 matched T2 cells [the TAP deficient line, provided by Dr. P. Cresswell] pulsed for 1 hr with 10 nM influenza virus matrix peptide [GILGFVFTL]. Target cells were labeled with Na<sup>51</sup>CrO<sub>4</sub> as previously described [Bhardwaj et al. 1994].

#### RESULTS

[0076] Cells Cultured in the presence of GM-CSF and IL-4 only were not fully mature. We first confirmed that GM-CSF and IL-4 induces the development of large numbers of potent APCs from lymphocyte-depleted PBMC when cultured for 6-7 days in the presence of FCS [ $2.5 \times 10^6$  per 40 ml of blood, Table 1 first row].

TABLE 1

Allogeneic MLR <sup>3</sup> H-TdR Incorporation, cpm x 10 <sup>-3</sup> AT T:APC ratios of:					
Treatment	100:1	300:1	900:1	DC Yield/40ml blood	% Enrichment
10% FCS	153 [17]	33 [3]	7	2.5 x 10 <sup>6</sup>	6%
10% FCS + PS	254 [196]	154 [135]	79	3.5 x 10 <sup>6</sup>	95%
10% FCS + CM	258 [55]	129 [27]	39	3.8 x 10 <sup>6</sup>	96%
1% Plasma	175 [3]	68 [1]	8	0.5 x 10 <sup>6</sup>	25%
1% Plasma + PS	309 [154]	161 [80]	56	2.0 x 10 <sup>6</sup>	75%
1% Plasma + CM	286 [8]	156 [1]	48	1.5 x 10 <sup>6</sup>	74%

Table 1: T cell stimulatory activity of dendritic cells derived from progenitors in blood

**[0077]** ER- cells were cultured for 7 days in RPMI medium containing GM-CSF and IL-4 and either 10% FCS or 1% human plasma. The cells were then cultured for 4 more days in the absence or presence of conditioned medium prepared from pansorbin or gamma globulin stimulated blood mononuclear cells [50% final concentration]. T cell stimulatory function was assessed in the primary allogeneic MLR. Results are shown as <sup>3</sup>H-TdR incorporation [cpm x 10<sup>-3</sup>] and are averages of triplicates. \* Numbers in parentheses represent cpm measured with syngeneic T cells. The yield is expressed as the total number of dendritic cells obtained from an original donation of 40 ml of blood. The % enrichment is defined as the percentage of cells in the non-adherent fraction that display typical features of dendritic cells: stellate shape, high expression of HLA-DR, the dendritic cell restricted marker CD83 [see Fig. 2].

**[0078]** We discovered, however, that dendritic cells primed under these conditions were not fully mature. Upon immunolabeling of cytopins or cell suspensions [FACS], there was residual expression of the monocyte markers CD14 and CD32 and weak to no expression of the dendritic cell restricted markers CD83 (Zhou et al. 1992; Zhou and Tedder, 1995) and p55 (Mosialos et al. 1996).

**[0079]** Furthermore, when FCS primed cells were recultured without cytokines, MLR stimulating activity decreased considerably and most of the cells attached firmly to plastic [data not shown]. These findings suggested to us that GM-CSF and IL-4 were insufficient triggers to generate mature and stable dendritic cells from non-proliferating blood progenitors.

**[0080]** When 10% human serum or plasma was used in the GM-CSF/IL-4 priming culture in the place of FCS, most of the PBMC remained adherent and few free dendritic cells developed by 7 days. To reduce adherence, medium containing 1% plasma or serum was tested instead. Some small floating aggregates gradually developed over 4 days, and at day 7, some dendritic cells were evident. Although these cells exhibited potent T cell stimulatory capacity in the allo MLR, [Table 1, fourth row], the yield of dendritic cells was low, only 0.5 x 10<sup>6</sup> cells per 40 ml of blood. As with FCS, the cells exhibited variable CD14 and CD32 expression and failed to express CD83 and p55. Thus human plasma was even less efficient than FCS in priming for mature dendritic cell development.

#### Requirement for conditioned medium

**[0081]** Two approaches were tested to increase dendritic cell yield and maturity in FCS and human plasma. A 1:10,000 dilution of Pansorbin was added to the cultures after 7 days of growth in GM-CSF and IL-4, and the cells were recultured for 3-4 days. Pansorbin increased the number of free floating dendritic cells as well as T cell stimulatory capacity in the allo MLR [Table 1, second and fifth rows]. However, there was a dramatic stimulation of syngeneic T cells possibly because of superantigens in the pansorbin.

**[0082]** In the second approach, we used conditioned medium [CM] derived from supernatants of T-depleted mononuclear cells that had been adhered to gamma globulin-coated plates or stimulated with pansorbin [see Methods]. On day 7 of culture, one half of the medium was replaced with this CM. At day 11, 4 days after the addition of CM, both the yield and enrichment of dendritic cells increased. [Table 1, rows 3 and 6]. There was also significant enhancement of allo stimulatory capacity, but without the increase in the syngeneic MLR seen with pansorbin. Thus we chose to use CM and 1% human plasma for all subsequent experiments. Plasma is preferable to use over serum since it is readily obtained during the Ficoll separation of blood.

**[0083]** In > 15 experiments, the overall effect of adding CM to plasma containing medium was to increase dendritic

cell yields 3-10 fold [range of  $0.3 - 3 \times 10^6$  per 40 ml of blood] and enhance enrichment from 2-25% to 30-90%. The contaminating cells are primarily lymphocytes, B cells and residual T cells. CM generated cells were phenotypically and functionally stable in that they retained their dendritic cell features when cultured in the absence of cytokines [see below]. No substantial difference was noted whether 33% vs. 50% CM was used or whether the CM was replenished every 2 days.

**[0084]** Throughout the above cultures, we noted the presence of residual adherent cells, most likely macrophages, in both components of the culture, i.e., during priming for 6-7d with GM-CSF and IL-4, and during maturation for 3-4d with CM. We therefore modified the protocol further. At day 7 of cytokine treatment, we directly transferred the nonadherent cells to fresh plates. We then cultured the cells for 4 additional days [day 11] in the absence or presence of CM. As expected, GM-CSF/IL-4 primed cells that were cultured without CM were suboptimal stimulators in the MLR [Figure 1, closed circles]. In contrast, there was a substantial increase in stimulatory activity when cells received CM, [open squares]. ER- cells that were cultured in Teflon beakers without exogenous cytokines throughout the 11 days were poor APCs [open circles].

#### Phenotypic analysis of dendritic cells grown from normal peripheral blood with GM-CSF, IL-4 and Conditioned Medium

**[0085]** The dendritic cells that were induced by this new culture procedure displayed a typical dendritic cell phenotype [Figure 2] and morphology [Figure 3]. The cells expressed high levels of MHC class II and accessory molecules for T cell stimulation [CD54, CD58, CD40, CD80, CD86]. The more lineage restricted antigens such as CD3, CD19, CD16, CD14 were absent. CD1a, CD4, CD11c and CD45RO were also expressed. CD68 or macrophage (Ramprasad et al. 1995; Rabinowitz and Gordon, 1991), was expressed in a perinuclear pattern. This pattern is distinct from that seen with macrophages, where staining is seen throughout the cytoplasm. Only rare cells stained with the anti-Lag mAb that identifies Birbeck granules in epidermal dendritic cells.

**[0086]** Two new markers, CD83 and p55, recently have been shown to be selectively expressed by the small subset of mature dendritic cells in cultured human blood (Zhou et al. 1992; Zhou and Tedder, 1995; Mosialos et al. 1995). Both markers were expressed only at low levels by cells that are primed with GM-CSF and IL-4, but were unregulated following culture in CM. CD83 is a member of the Ig superfamily, and p55 is an EBV-inducible actin bundling protein in B cells (Zhou et al. 1992; Zhou and Tedder, 1995; Mosialos et al. 1995).

#### Stability of dendritic cells generated in GM-CSF/IL-4 and CM

**[0087]** The stability of the dendritic cells generated at various points during the two step culture protocol described above was also ascertained. ER- cells were cultured with GM-CSF/IL-4 for 7 days and then analyzed for stimulatory activity in an allo MLR. Although these cells could stimulate allogeneic T cells [Table 2, first row], the phenotype was not typical of mature DCs [CD14+, moderate levels of HLA-DR, little to no CD83, and a diffuse staining for CD68; data not shown].

TABLE 2

Treatment				Allogeneic MLR		
Culture supplements on days:				<sup>3</sup> H-TdR Incorporation (cpm x 10 <sup>-3</sup> ) at T:APC ratio of:		
0-7	7-11	11-14	Day of MLR test	100:1	300:1	900:1
GM + IL-4	-	-	7	161	145	81
"	no CM	-	11	203	146	25
"	+ CM	-	11	154	172	161
"	+ CM	no CM	14	144	170	189
"	+ CM	+ CM	14	178	207	165

Table 2: CM is required for optimal and stable immunostimulatory activity

**[0088]** ER- cells were cultured in GM/CSF and IL-4 for 7 days, washed and then analyzed immediately for stimulatory activity in an allo MLR [first row], or cultured for 4 more days in the absence or presence of CM [second and third rows]. At day 1, aliquots of dendritic cells were maintained in CM for three more days [row 4] or washed extensively and recul-

tured in the absence of CM [row 5]. The same donor of allogeneic T cells was used throughout these experiments. Results are shown as  $^3\text{H-TdR}$  incorporation [ $\text{cpm} \times 10^{-3}$ ] and are averages of triplicates.

[0089] Cells washed out of cytokines at day 7 and recultured for 4 more days in the absence of CM had a similar phenotype and a noticeable decline in T cell stimulatory function when compared with dendritic cells grown in GM-CSF/IL-4 and CM [compare rows 2 and 3, Table 2]. Cells were next washed out of CM at day 11 and cultured for three more days [day 14] alongside cells that were left in CM. The dendritic cells were identical with respect to their phenotype and stimulatory capacity in the allogeneic MLR [rows 4 and 5, Table 2]. Thus, the exposure to CM renders an apparent irreversible change that leads to functionally mature dendritic cells.

#### Attempts to identify the active cytokines in CM

[0090] The CM from blood cells that are cultured in human gamma globulin likely contain many monocyte-derived cytokines such as IL-1, IL-6 and  $\text{TNF}\alpha$ . However, none of these cytokines could replace the CM, and in fact each cytokine had no effect on increasing dendritic cell yield or function [Figure 4 and Table 3]. The cytokines IL-12 and IL-15 were also ineffective [Table 3].

TABLE 3

Treatment			Allogeneic MLR		
Culture supplements on day 7			$^3\text{H-TdR}$ Incorporation ( $\text{cpm} \times 10^{-3}$ ) at T:APC ratio of:		
CM	Other		100:1	300:1	900:1
+	goat IgG	[4 $\mu\text{g}/\text{ml}$ ]	147	117	46
+	goat IgG	[17 $\mu\text{g}/\text{ml}$ ]	141	94	31
+	$\alpha\text{TNF-}\alpha$	[1 $\mu\text{g}/\text{ml}$ ]	144	120	61
+	$\alpha\text{IL-6}$	[4 $\mu\text{g}/\text{ml}$ ]	161	120	31
+	$\alpha\text{IL-12}$	"	180	104	43
+	$\alpha\text{IL-1-}\alpha$	"	152	90	33
+	$\alpha\text{IL-1-}\beta$	"	163	112	37
+	cocktail	[17 $\mu\text{g}/\text{ml}$ ]	116	59	19
-	$\text{TNF-}\alpha$	[10 $\text{ng}/\text{ml}$ ]	79	38	11
-	IL-1- $\beta$	"	72	47	4
-	IL-12	[500 $\text{pg}/\text{ml}$ ]	55	21	4
-	IL-15	"	46	12	4

Table 3 : Attempts to identify the factors in CM that promote dendritic cell differentiation

[0091] ER- cells were cultured in GM/CSF and IL-4 for 7 days, and then supplemented with CM for 4 days in the presence of neutralizing antibodies to a panel of cytokines. Goat IgG was the control. Cocktail refers to the combination of the various antibodies. Some cultures were supplemented with recombinant cytokines alone in the absence of CM. At day 11 of culture the cells were evaluated for their ability to stimulate allogeneic T cells. Results are shown as  $^3\text{H-TdR}$  incorporation [ $\text{cpm} \times 10^{-3}$ ] and are averages of triplicates.

[0092] We also added polyclonal neutralizing antibodies to cytokines to the CM. However, antibodies to  $\text{TNF}\alpha$ , IL-1, and IL-6 did not block the capacity of CM to increase dendritic cell yields and function [Figure 4 and Table 3].

#### Source of DC progenitors in PBMC

[0093] The progenitor cells that give rise to dendritic cells produced according to an embodiment of the invention [GM-CSF + IL-4 followed by CM] were characterized. To assess the adherence of progenitors to plastic, ER- cells were adhered for 1 hour in 6 well plastic plates [ $2 \times 10^6$  per well] after which nonadherent cells were removed and replated in new dishes. Both populations were cultured in GM-CSF and IL-4 for 7 days followed by CM for 4 days. Adherent ER-

cells yielded the highest number and purity of dendritic cells [Table 4]

TABLE 4

DC progenitors are present in both adherent and non-adherent ER - fractions.					
			Allogeneic MLR		
			<sup>3</sup> H-TdR incorporation (cpm x 10 <sup>-3</sup> ) at T:APC ratio of:		
Cell population	DC Yield/40ml blood	% Enrichment	100:1	300:1	900:1
ER -	0.8 - 1.5 x 10 <sup>6</sup>	79 - 90	265	243	169
ER -, adherent	1.3 - 2.8 x 10 <sup>6</sup>	94 - 96	226	222	147
ER -, nonadherent	0.8 - 1.7 x 10 <sup>5</sup>	27 - 36	219	192	73

Table 4: Dendritic cell progenitors are present in both adherent and nonadherent ER- fractions

[0094] ER- cells were adhered to plastic for 1 hr. Both adherent and nonadherent cells were collected and cultured with GM-CSF and IL-4 for 7 days, followed by CM. At day 11, the yield and % enrichment of dendritic cells was determined. The T cell stimulatory function of the ER-populations analyzed is also shown. Results are representative of 2 experiments and were similar to cells derived from bulk ER- cells in terms of immunostimulatory capacity. ER- nonadherent cells also generated some potent APCs, but the yield and purity of dendritic cells was substantially less, primarily because of a large number of contaminating lymphocytes. Thus progenitors appear to reside in both adherent and non-adherent populations of blood mononuclear cells.

[0095] ER- cells were stained with PE-conjugated anti-CD14 and the cells were sorted in a FACStar into CD14 high and CD14 low fractions. Negative small cells were excluded by gating. These were then cultured for 7 days with GM-CSF and IL-4 followed by 4 days in CM. CD14 high cells were also evaluated with or without prior treatment with ionizing irradiation [Gy 15 or 30]. The greatest yield, percent enrichment and T cell stimulatory activity were found in the unirradiated CD14 high population [Table 5 and Figure 5].

TABLE 5

DC progenitors are enriched in the CD14 hi population		
Cell population	Yield/40ml blood	% Enrichment
CD14 hi	1.2 - 2 x 10 <sup>6</sup>	66 - 97
CD14 lo	0.7 - 0.8 x 10 <sup>6</sup>	10 - 39
CD14 hi, XRT	1 - 1.6 x 10 <sup>6</sup>	72 - 77
CD14 hi + lo	1 - 1.3 x 10 <sup>6</sup>	57 - 73
[n = 2]		

Table 5: Dendritic cell progenitors are enriched in the CD14 hi population

[0096] ER- cells were stained with CD14-FITC and sorted into CD14 hi and dim populations. Separated fractions, irradiated CD14 hi cells [Gy 15 and 30] and stained unsorted or combined cells [CD14 high + low] were cultured with GM-CSF/IL-4 followed by CM. The yields and %enrichment of dendritic cells is shown.

[0097] Interestingly, irradiated CD14+ high cells generated effective dendritic cells but the yield was 50% lower, and the percent enrichment was slightly lower, 77% vs. 97% for nonirradiated CD14+ cells. The CD14 low fraction also produced some typical dendritic cells, but the yield and enrichment were significantly lower than the CD14 high population, probably because there is a significant number of lymphocytes contaminating this fraction.

[0098] Collectively, our data suggest that dendritic cell progenitors are found in PBMC populations that are both plastic adherent and nonadherent, as well as CD14 dim and CD14 high. Furthermore, some of these progenitors are radioresistant.

Dendritic cells derived from non-proliferating progenitors induce strong anti-viral cytolytic T cell responses

[0099] Potent human CD8<sup>+</sup> cytolytic T cell [CTL] responses to live replicating influenza A virus are generated when dendritic cells are the APCs (Bhardwaj et al. 1994). When pulsed with poorly replicating, heat-inactivated, influenza virus, dendritic cells induce equally strong CTL responses (Bender et al. 1995). Other APCs, e.g., macrophages lack this capacity. Since the heat inactivated virus is incapable of substantial new protein synthesis, only small amounts of viral protein are required to charge class I molecules on dendritic cells. Dendritic cells prepared as described above from non-proliferating progenitors in blood were tested for their capacity to elicit anti-viral CTL responses to live and heat-inactivated influenza virus [Table 6].

TABLE 6

Percent specific lysis of targets			
Infection of dendritic cells	Mo [-]	Mo [Flu]	
[-]	2.5	1.9	
Live Flu	1.3	60.4	
Infection of dendritic cells	T2 [-]	T2 [Matrix peptide]	
[-]	0	10	
Heated Flu	30	77	

Table 6: Dendritic cells derived from non-proliferating progenitors induce strong anti-viral cytolytic T cell responses

[0100] Dendritic cells were uninfected or infected with live replicating or heat inactivated influenza virus [see methods] and cocultured with autologous HLA A2.1<sup>+</sup> T cells [T:APC ratio = 30:1].

[0101] After 7 days, T cells were harvested and tested for cytolytic activity on chromium labeled syngeneic macrophages [mos] which were infected or uninfected with influenza virus [upper panel]. Lytic activity of T cells responding to dendritic cells infected with heat inactivated influenza virus was tested on chromium labeled T2 target cells [lower panel] that had been pulsed with 10 nM of HLA A2.1 restricted matrix peptide. E/T ratio in both experiments was 30:1.

[0102] Both forms of virus could be presented to autologous T cells to induce CTL responses. CD8<sup>+</sup> T cells are the likely mediators, since they efficiently lysed TAP deficient T2 cells pulsed with the HLA A2.1 restricted influenza matrix peptide.

Conditions for generating mature dendritic cells from progenitors in human plasma

[0103] The method of preparing mature dendritic cells according to this invention preferably uses two steps. Two steps are preferred because dendritic cells generated in either FCS or human plasma containing medium failed to develop and maintain a stable phenotype and function when removed from GM-CSF and IL-4. For example, these "putative" dendritic cells continued to express CD14 and CD32, which are typically absent from mature dendritic cells, expressed little or no CD83 and p55 and lost stimulatory function upon removal of GM-CSF/IL-4. This phenomenon has not been previously appreciated, since the cells are typically used without reculturing them in the absence of cytokines (Sallusto and Lanzavecchia, 1995; Sallusto and Lanzavecchia, 1994). Second, exposure to a dendritic cell maturation factor such as CM was an absolute requisite to induce the formation of large numbers of fully differentiated dendritic cells from the GM-CSF and IL-4 primed cultures. The CM-induced cells expressed a typical phenotype, with strong expression of a) antigen presenting MHC class I and II products and CD1a, b) several adhesions and co-stimulator molecules including CD40, CD54, CD58, CD80 and CD86, and c) two dendritic cell restricted molecules, CD83 and p55. This phenotype remained stable following removal of all growth factors. Moreover, the yield and T cell stimulating potency of the CM-treated cells were substantial. Up to  $3 \times 10^6$  dendritic cells were obtained from 40 ml of blood, which is 3 times more than the number of mature dendritic cells that are present in one unit [500 ml] of non treated blood cells. The cells induced strong allo MLRs even at DC:T ratios of 1:900. It is preferred to first prime the blood progenitors with GM-CSF and IL-4 for 6-7 days before adding the CM, since the latter seems to be less effective if added at day 0 [data not shown].

Dendritic cell derived from blood progenitors express a unique pattern of antigens

**[0104]** We find that CD83 and p55 are useful markers for the development of mature dendritic cells from blood progenitors. Both antigens are uniformly expressed at high levels in mature cells but are lacking in fresh blood monocytes and in progenitors that are primed in GM-CSF/IL-4 for 7 days but are not matured in CM. Another useful marker to distinguish dendritic cells from monocytes is CD68 [termed macrosialin in the mouse], a member of the lamp-1 family. This antigen is detected in a perinuclear area in dendritic cells, whereas much of the cytoplasm stains for CD68 in monocytes, as has been emphasized by Gordon and others (Rabinowitz and Gordon, 1991; Ramprasad et al. 1995).

EXAMPLE 2Materials And MethodsCulture medium

**[0105]** The following culture media were used: RPMI 1640 [Biological Industries, Kibbutz Beit Haemek Israel]; X-VIVO10, 15, and 20™ [Bio-Whittaker, Walkersville, MD]; HybriCare and AIM-V™ [GIBCO-BRL, Gaithersburg, MD]. They were supplemented with 50 µg/ml of gentamicin. Sources of serum were FCS [10%, Biological Industries or Seromed-Biochrom, Berlin, Germany], and human plasma and serum [0.5, 1, 5, and 10%] from either adults [PAA Laboratories GmbH, Lin, Austria, blood bank or autologous] or from umbilical cord blood [kindly provided by the Department of Gynecology and Obstetrics]. FCS and, in some experiments only, human sera/plasma was heat-inactivated at 56°C for 30 minutes.

Cytokines and anti-cytokines

**[0106]** Recombinant human GM-CSF was generously provided by Dr. E. Liehi, Sandoz Ges.m.b.H., Vienna, Austria [specific activity  $5.9 \times 10^6$  U/mg]. Alternatively, human GM-CSF prepared for therapeutical purposes was used [Leukomax™, Sandoz, Basel, Switzerland, specific activity  $1.1 \times 10^7$  U/mg]. Recombinant human IL-4 was a gift of Dr. M.B. Widmer, Immunex Corp., Seattle, WA [specific activity  $5 \times 10^7$  U/mg] and Dr. E. Liehl, Sandoz, TNF-α was supplied by Dr. G.R. Adolf [Bender, Vienna, Austria; specific activity  $6 \times 10^7$  U/mg]. IL-12 was provided by the Genetics Institute, Boston, MA [specific activity  $3.6 \times 10^8$  U/mg]. Human IL-1β [specific activity  $5 \times 10^8$  U/mg] and simian IL-15 [specific activity  $1 \times 10^7$  U/mg] were purchased from Genzyme Corp., Cambridge, MA human stem cell factor [specific activity  $0.5\text{-}1.0 \times 10^6$  U/mg] from R&D Systems [MO]. Human IL-15 and a neutralizing rabbit anti-human IL-15 antibody were from PeproTech, London, UK. Human IL-13 was a gift of Dr. A. Minty, Sanofi, Labège, France and was also obtained from Pharmingen, San Diego, CA [specific activity  $1 \times 10^6$  U/mg].

Initial processing of human blood

**[0107]** Peripheral blood was obtained from the local blood bank as standard buffy coat preparations and from normal donors. Preservative-free heparin [200 I.U./ml blood; Novo Nordisk A/S, Bagsvaerd, Denmark] was used to prevent clotting. PBMC were isolated by centrifugation in Lymphoprep [1.077 g/ml; Nycomed Pharma AS, Oslo, Norway]. In order to minimize contamination of PBMC with platelets the Lymphoprep centrifugation [200 x g/room temperature] was interrupted after 20 minutes. The top 20-25 ml containing most of the platelets were carefully removed and centrifugation was resumed [20 minutes/460 x g/room temperature]. PBMC were then depleted of T and B cells by means of an immunomagnetic technique. Dynabeads [Dyna, Oslo, Norway] M-450 Pan-B/CD 19 and M-450 Pan-T/CD2 were washed four times with PBS containing 1% bovine serum albumin or human plasma. PBMC [ $30\text{-}50 \times 10^6$ /ml] were incubated with the magnetic beads at a ratio of 1:1:1 at 4°C for 20 min. using Dynal mixer. Lymphocytes were then depleted by means of a Dynal magnet [1-2 minutes]. Magnet-non-adherent cells were harvested and washed. This depletion step was repeated once. In some experiments an indirect immunomagnetic approach was used. PBMC were labeled with anti-T and B cell mAb's [hybridoma supernatant of mAb OKT-11/CD2 and ascitic fluid of mAb MEM-97/CD20; gift of Dr. v. Horejsi, Praha, Czech Republic], washed three times and incubated with sheep anti-mouse Ig-coupled magnetic beads [SAM-M450; Dynal] at a ratio of 3 beads to one cell. Magnetic separation was achieved as described above. In addition, lymphocyte-depleted PBMC were obtained from cancer patients [after informed consent] in complete remission during hematopoietic recovery after high-dose consolidation chemotherapy and subcutaneous daily administration of 300 µg G-CSF [Neupogen™, Hoffmann-La-Roche, Basel, Switzerland]. Depletion of PBMC from CD34+ cells was achieved either by means of M-450/CD34 magnetic beads [Dyna] or by passing cells through an immunoaffinity anti-CD34 column [Cellpro Inc., Bothell, WA] that is clinically used for transplantation purposes. PBMC from normal donors treated with G-CSF in the same manner were also used. This study was approved by the Ethical Committee of the Medical Fac-

ulty, University of Innsbruck.

#### Culture technique

- 5 [0108] Lymphocyte-depleted PBMC were planted in 6-well tissue culture plates at a density of  $2 \times 10^6$  cells/well in 3 ml of complete culture medium. GM-CSF and IL-4 were added at final concentrations of 800 and 1000 U/ml, respectively. Cultures were fed every other day [days 2, 4 and 6] by removing 1 ml of the medium and adding back 1.5 ml fresh medium with cytokines [1600 U/ml GM-CSF and 1000 U/ml IL-4, resulting in final concentrations of 800 and 500 U/ml, respectively]. On day 7, non-adherent cells were either harvested and analyzed or transferred to new 6 well plates and
- 10 cultured further in the presence or absence of maturation stimuli [see below]. GM-CSF and IL-4 were present during the culture period from day 7 to day 10-11.

#### Stimuli for dendritic cell maturation

- 15 [0109] SACS [fixed Staphylococcus aureus Cowan 1 strain, 2.01 mg/ml Ig-binding capacity, Pansorbin cells, Cat No. 507861] was purchased from CalBiochem Co., La Jolla, CA. It was added to dendritic cell cultures at a final dilution of 1:10000. Conditioned medium was produced by Ig-adherent PBMC. To this end, bacteriologic plates [100 mm. Cat. No. 1029, Falcon, Oxnard, CA] were incubated with a solution of human Ig [10 mg/ml, therapeutically used "Immunoglobulin i.v. Biochemie", Biochemie Ges.m.b.H., Vienna, Austria, subsidiary of Sandoz] in PBS for 1 minute at room tempera-
- 20 ture. After three rinses with PBS  $5 \times 10^7$  PBMC were put into one Ig-coated bacteriologic plate for 1 hour at 37°C in 8 ml of complete culture medium. Non-adherent cells were rinsed away and adherent cells were cultured in fresh complete medium at 37°C for 24 h.
- [0110] Complete media consisted of the different media and serum supplements mentioned above. Conditioned media were sterile filtered and stored at +4°C for one week at the longest or frozen at -20°C. In some cases, conditioned
- 25 medium was prepared from cells stimulated with SCS instead of human Ig. Conditioned media were routinely used at 25% vol/vol. Increasing this concentration up to 50% was not appreciably better.

#### Flow cytometry and immunohistochemistry

- 30 [0111] Primary antibodies used for flow cytometric and immunohistochemical analyses are listed in Table 7.

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TABLE 7

Antibodies used for immunohistochemistry				
Specificity	Clone/Name	Ig class	Source	Reference
HLA-DR/DQ	9.3F10/HB 180	mouse IgG2a	ATCC <sup>a</sup>	
HLA-DR	L243/HB55	mouse IgG2a	ATCC	
HLA-DR-FITC	L243	mouse IgG2a	BDIS <sup>b</sup>	
CD1a	OKT-6/CLR8020	mouse IgG1	ATCC	
CD1a-FITC	OKT-6	mouse IgG1	Ortho <sup>c</sup>	
CD15s	CSLEX1	mouse IgM	BDIS	
CD40	G-28.5	mouse IgG1	E.A. Clark <sup>d</sup>	
CD44v9	VFF16	mouse IgG	P.Herrlich <sup>e</sup>	
CD45RA	4G10	mouse IgG2a	R.M. Steinman <sup>f</sup>	
CD45RO	UCHL10	mouse IgG2a	DAKO <sup>g</sup>	
CD68	EBM11	mouse IgG1	DAKO	
CD71	RPN.511	mouse IgG1	Amersham <sup>h</sup>	
CD80	L307.4	mouse IgG1	BDIS	
CD83	HB-15a	mouse IgG2b	T.F. Tedder <sup>i</sup>	
CD86	IT2.2	mouse IgG2b	Pharmingen <sup>j</sup>	
CD115/c-fms	Ab-1	rat IgG1	Oncogene	
CSF-1-receptor	(clone 2-4A5-4)	rat IgG2b	Science <sup>k</sup>	
Birbeck granules	Ab-2 (clone 3-4A4-E4)	mouse IgG1	S. Imamura <sup>l</sup>	
Lag				
Proliferation-associated	Ki-67	mouse IgG1	DAKO	

<sup>a</sup> American Type Culture Collection, Rockville, MD;

<sup>b</sup> Becton-Dickinson Immunocytometry Systems, Mountain View, CA;

<sup>c</sup> Ortho, Raritan, NJ;

<sup>d</sup> Seattle, WA;

<sup>e</sup> Kernforschungszentrum, Karlsruhe, Germany;

<sup>f</sup> The Rockefeller University, New York, NY;

<sup>g</sup> Roskilde, Denmark;

<sup>h</sup> Amersham, UK;

<sup>i</sup> Duke University, Durham, NC;

<sup>j</sup> San Diego, CA;

<sup>k</sup> Cambridge, MA;

<sup>l</sup> Kyoto University, Kyoto, Japan

**[0112]** Secondary antibody was biotinylated anti-mouse or anti-rat Ig's followed by FITC-or PE-conjugated streptavidin [Amersham International, Amersham, UK]. Dead cells were gated out on the basis of their fluorescence with propidium iodide or by their light scatter properties. Analyses were done on a FACScan instrument [Becton-Dickinson, Mountain View, CA]. Cytospins were prepared with Cytospin 2 centrifuge [Shandon, Inc., Pittsburgh, PA]. Slides were acetone-fixed for 5 min. at room temperature and then incubated with primary mAbs for 15 minutes followed by biotinylated sheep anti-mouse Ig [1:200; Amersham] and Texas Red-conjugated streptavidin [1:50, Amersham]. For double labeling purposes this staining sequence was extended with a blocking step [100 µg/ml mouse gamma globulin] and FITC-conjugated anti-HLA-DR [Becton-Dickinson]. Slides were mounted in Vectashield™ [Vector Laboratories, Burlingame, CA]

Allogenic MLR and processing/presentation of soluble antigen

**[0113]** To test for T cell stimulatory function, the APCs were irradiated with 30 Gy from a Cs source and added in graded doses as simulators for  $2 \times 10^5$  purified [nylon wool-non adherent; anti MHC class II-panned], allogeneic T cells in 96 well flat bottomed plates [Falcon]. For some experiments T cells were purified from umbilical cord blood in order to obtain naive T cells. Proliferation was determined on days 4-6 with the addition of 4  $\mu$ Ci-148 KBq/ml of [ $^3$ H]TdR [specific activity 247.9 GBq/mmol=6.7 Ci/mmol; New England Nuclear, Boston, MA] for 12-16h to triplicate wells [mean cpm]. Processing and presentation of tetanus toxoid protein [Connaught, Willowdale, Ontario, Canada] was measured with a HLA-DP4- restricted tetanus-peptide-specific T cell clone [AS11.15] that was a gift of Dr. A. Lanzavecchia, Basel Switzerland [Lanzavecchia, 1985]. Graded doses of irradiated dendritic cells were co-cultured with  $1.5 \times 10^4$  clone cells in the presence or absence of tetanus toxoid [5 and 1  $\mu$ g/ml]. [ $^3$ H] TdR was added from d2 to d3.

**[0114]** To generate cytotoxic T cells, the approach described by (De Bruijn et al. 1992) was used with modifications. Irradiated [12.5 Gy] dendritic cells as simulators were co-cultured with autologous PBMC or with CD8+ T lymphocytes [1:25] as responders in the presence of 100-200  $\mu$ M peptide MP 58-66 of influenza matrix protein [MedProbe, Oslo, Norway] in 2ml RPMI/10% human serum in wells of a 24-cell tissue culture plate. After one week the cultures were fed with 40% Lymphocult [Biotest, Dreieich, Germany]. The lytolytic potential of outgrowing T cells was assessed two to four weeks after the start of the cultures in a standard Chromium-release cytotoxicity assay using as target PHA-stimulated autologous T cell blasts, that had been incubated with different concentrations of the peptide. Results are expressed as % specific lysis. CD8+ T lymphocytes were prepared by indirect immunomagnetic depletion of CD4+ T cells using mAb VIT-4/anti-CD4 [gift of Dr. W. Knapp, Vienna, Austria] followed by sheep anti-mouse IgG magnetic beads [Dynal].

Additional reagents

**[0115]** Latex beads [0.5% vol/vol; 2  $\mu$ m diameter] for phagocytosis experiments were purchased from Seradyn, Indianapolis, IN. They were added to the cell cultures at a final dilution of 1:20. Staphylococcal enterotoxin A [superantigen SEA] was from Sigma Chemical Corp. St. Louis, MO. It was used at a final concentration of 25  $\mu$ g/ml. LPS [Sigma] was added to some experiments at a final concentration of 100 ng/ml.

RESULTSIdentification of culture conditions allowing full maturation of dendritic cells

**[0116]** Dendritic cells grown in GM-CSF and IL-4 are not fully mature. Lymphocytes were depleted from PBMC by means of immunomagnetic beads. Use of the immunomagnetic beads allowed for the omission of a sensing step two hours after plating. Dendritic cells developed from these lymphocyte-depleted PBMC in the same way as from whole PBMC populations.

**[0117]** Dendritic cells, that had been washed out of GM-CSF and IL-4 and were replated in culture medium without cytokines for an additional three days [days 7-10], lost their characteristic morphology and re-adhered. They assumed the typical shapes of macrophages [Figure 6A]. In addition, dendritic cells on d7 of culture expressed the macrophage marker CD 115 [c-fms/CSF-1 receptor] although their CD14 expression was very low or absent. In contrast, they did not express CD83, a marker for mature dendritic cells [Zhou and Tedder, 1995] [Figure 7]. The staining pattern of a well defined population of mature dendritic cells, namely cutaneous dendritic cells obtained by emigration from skin organ cultures [Pope et al. 1995] was inverse: CD115/CD83+ [not shown]. From this we concluded that dendritic cells grown in GM-CSF and IL-4 were not fully and irreversibly mature although they did express some maturation markers such as high levels of MHC class II, furthermore CD40, 54, 58, 80 and a strong T cell stimulatory capacity [Romani et al. 1994; Sallusto and Lanzavecchia, 1994].

Determination of stimuli for full maturation of dendritic cells

**[0118]** We tested whether full maturation could be brought about by adding cytokines to the cultures. Supplementation of GM-CSF and IL-4 containing culture medium with IL-1 $\beta$  [50U/ml] and/or TNF- $\alpha$  [50U/ml] either from day 0 through to d7 or as a 24-hour pulse from day 0 to day 1 or day 6 to day 7 increased immunostimulatory capacity as expected but did not lead to a stable phenotype of mature dendritic cells as defined above. Addition of these cytokines after the initial 7-day culture and transfer of the cells to fresh culture wells [i.e., from day 7-10] gave the same result [Figure 8]. Similar findings were made for the cytokines IL-12 [1 ng/ml], stem cell factor/c-kit ligand [yyU/ml] and IL-15 [20ng/ml].

**[0119]** SACS [fixed Staphylococcus aureus], a potent natural stimulator of cytokine secretion was also tested. When a 1:10,000 dilution of SACS was added to the cultures from day 7 to day 10 or 11 of culture, the numbers of free floating

dendritic cells increased markedly. Moreover, these cells displayed a more pronounced morphology with highly motile cytoplasmic processes ["veils"]. The cells also did not re-adhere when replated without cytokines. T cell stimulatory capacity in the allogeneic MLR was superior to dendritic cells that had been cultured without SACS [Figure 8]. However, there was a dramatic stimulation of syngeneic T cells possibly because of superantigens in the SACS preparation. Addition of superantigen to the cultures between days 7 to 10 did not bring about maturation of dendritic cells. However, supernatants of SACS-stimulated adherent PBMC [i.e., conditioned media] had the same effect as SACS itself.

**[0120]** Conditioned medium [CM] was produced in a way that could potentially be of clinical use and avoid the involvement of bacterial products such as SACS. Therefore, we derived supernatants from whole or T-depleted nonnuclear cells that had been stimulated by adherence to Ig-coated plates. On day 6 to 8 [mostly 7] of culture dendritic cells were transferred to fresh wells and 25% vol/vol of CM was added. Three to four days later the cells were analyzed. There were almost no adherent cells left on the bottom of the wells. By phase contrast microscopy revealed that the floating cells had many motile veils, much like SACS-treated cells [Figure 9]. In the hemocytometer the cells appeared "hairy" like cultured epidermal Langerhans cells or cutaneous emigrant dendritic cells. This is in contrast to either 7 day or 10 day cultures without CM, where the cells are large with irregular outlines, but the number of long processes and veils is low. Cultured without cytokines the CM-exposed cells remained stably non-adherent over an observation period of 3 days [Figure 6B-D]. They now expressed CD83 and were highly stimulatory for resting T cells [see below]. Therefore, dendritic cells generated in the presence of monocyte CM qualified as fully mature dendritic cells. In >15 experiments the yields of mature, CD83-expressing dendritic cells per 40 ml of blood ranged between 1 and 4 x 10<sup>6</sup> in FCS-containing media. Maturation occurred likewise when IL-4 was replaced with IL-13 [20 ng/ml].

**[0121]** To ascertain whether the activity of CM is due to IL-15, a neutralizing antibody was added to CM during the maturation phase of culture. Maturation was not prevented or impaired. We also measured whether CM contained bioactive IL-15. IL-2-responsive CTLL-2 cells also proliferated vigorously in response to both human and simian IL-15 in concentrations as low as 5 pg/ml. No response was seen when CM were tested in this assay [n=2] indicating that IL-15 is present only in minute quantities if at all [Table 8].

TABLE 8

Test for IL-15 in the monocyte conditioned medium				
Additions to the CTLL bioassay for IL-15	no antibody	anti-IL-5 1:100	anti-IL-15 1:200	anti-IL-15 1:100 + excess IL-15
no cytokines	0.2			
IL-15 [500 pg/ml]	152.8	148.8	161.1	149.2
IL-15 [50 pg/ml]	151.1	145.3	147.0	144.2
IL-15 [5 pg/ml]	45.8	0.9	0.8	156.9
monocyte conditioned medium [25% vol/vol]	0.6	2.4	1.1	155.0

**[0122]** Table 8: IL-15-dependent proliferation of CTLL-2 cells was determined by measuring <sup>3</sup>H-TdR incorporation during the final 6 hours of a 36 hour culture [6 x 10<sup>3</sup> CTLL cells per well]. Values are means of triplicate wells and are given in cpm x 10<sup>-3</sup>. Excess IL-15 was added at a final concentration of 1 ng/ml.

#### Development of FCS-free media

**[0123]** Having established the conditions for the full maturation of dendritic cells it was desirable to develop a system without fetal calf serum. Simply replacing FCS with human pool sera resulted in little or no development of dendritic cells. We observed increased adherence of cells to the culture surfaces. Serum, plasma and serum from umbilical cord blood were tested over a concentration range from 0.5 to 10%. We also compared autologous sera/plasma versus pooled sera/plasma from the blood bank or commercially available reagents. The effects of heat-inactivation were tested. These variables were combined with different culture media such a RPM1640, X-VIVO, AIM-V, Hybricare, and Iscove's. These reagents were used for the culture of both progenitor cells and CM-producing adherent PBMC. The primary read-out system was morphology under the inverted phase contrast microscope and the yield of mature, stable dendritic cells. Cultures in RPMI supplemented with 10% FCS were always run in parallel as a positive control. RPMI1640 containing 1% autologous, not heat-inactivated human plasma was observed to be optional. In 12 standardized experiments the yield ranged between 0,8 to 3,3 x 10<sup>6</sup> CD83+ dendritic cells from 40 ml of blood. Enrichment of

CD83+ cells was 30 to 80%.

#### Properties of mature dendritic cells

5 [0124] Phenotype. FACS analyses were performed before and three days after exposure to CM. Alternatively, dendritic cells were cultured from day 7 to day 10 in the presence or absence of CM and then compared to each other. Figure 7 shows expression of the key markers: CD83 is induced, CD86 is enhanced, and CD115 is lost. This pattern applies also to dendritic cells cultured in clinically approved media and derived from PBMC that have been depleted from lymphocytes by immunomagnetic beads or sheep erythrocyte rosetting. CD14 is low or absent and CD1 shows some reduction in expression levels upon maturation. Further CM-induced phenotypical changes include the down-regulation of CD32 and CD45RA and the upregulation of CD45RO. Substantial levels of CD4 are expressed on both immature and mature dendritic cells. Staining of cytocentrifuge smears [not shown] also reflected the maturation event that takes place under the influence of CM. Dendritic cells on day 7 of culture are stained throughout the whole cytoplasm in a pattern typical for monocytes or macrophages. After three days of culture in the presence of CM the immunostained cellular structures become concentrated in a dull spot near the nucleus. This pattern has been described for mature dendritic cells. MAb's Lag and Ki-67, specific for Birbeck granules of epidermal dendritic cells [i.e., Langerhans cells] and a proliferation-associated antigen, respectively, stained only rare dendritic cells.

10 [0125] Function. One of the mechanisms by which dendritic cells take up antigens is phagocytosis. This function is down-regulated upon culture of dendritic cells (Reis e Sousa et al. 1993). We tested the phagocytic capacity of dendritic cells before and after exposure to CM. Immature dendritic cells readily took up 2µm latex beads; mature dendritic cells after three days of culture in CM phagocytosed only very few particles [Figure 10].

15 [0126] Maturation in conditioned medium enhanced the stimulatory capacity for resting T cells markedly (Figures 11A, 11B). Cord T cells, i.e., naive T cells were also efficiently stimulated by mature dendritic cells [Figure 11C]. When we tested for the capacity to process and present a soluble native protein antigen [tetanus toxoid] using a tetanus peptide-specific T cell clone a reciprocal pattern emerged. Dendritic cells that had been cultured in the presence of CM were less efficient than those cultured in the absence of CM [Figure 11D] indicating maturation (Sallusto and Lanzavecchia, 1994; Romani et al. 1989a). Mature dendritic cells, when pulsed with a dominant influence matrix peptide that is presented on HLA-A2.1 were able to elicit peptide-specific, MHC class I-restricted, lytic T cells lines from autologous PBMC or populations of CD+ T cells [Figure 11E, F].

#### Modifications of the method with regard to clinical applicability

20 [0127] With regard to the clinical application of cultured dendritic cells we tested whether the method would work in the complete absence of xenoproteins and with culture media that are approved for clinical use. Comparative data showed that depletion of lymphocytes [T and B] with immunomagnetic beads was equivalent to the commonly used method of rosetting with neuraminidase-treated sheep erythrocytes discussed above. Similar numbers of stimulatory dendritic cells were obtained. Anti-B and T cell mouse mAb's and immunomagnetic sheep anti-mouse Ig beads, both approved for clinical use are available from Baxter Healthcare Corp. [Glendale, CA]. Culture media for clinical use are optimized for the growth of cell types other than dendritic cells [e.g. tumor-infiltrating lymphocytes]. Yet, in more than 15 experiments mature dendritic cells could consistently be procured. When X-VIVO 20 medium supplemented with 1% autologous human plasma was used for both culturing the cells and producing CM, substantial numbers of stimulatory dendritic cells could be obtained. Yields and percentages of enrichment were, however, only about half of what could be achieved with RPMI 1640 medium [n=5]. More adherent cells were observed in these cultures. Pilot experiments with AIM-V medium gave similar results. X-VIVO 10 and X-VIVO 15 media yielded unsatisfactory results.

45 [0128] With regard to the possibility that only small volumes of blood may be available in certain clinical settings we determined that pretreatment of donors with G-CSF increased dendritic cell yields. Blood from G-CSF-treated cancer patients and normal individuals gave up to 6-fold yields of mature [i.e., CD83+] dendritic cells. This increase was not only due to an increased frequency of CD34+ precursor cells in the blood because similar numbers of immunostimulatory mature dendritic cells could be grown both from CD34-containing and CD34-depleted PBMC starting populations in response to GM-CSF, IL-4 and CM [Figure 12].

50 [0129] We also tested whether cryopreservation of dendritic cells precursors and their progeny is possible. Indeed, using standard freezing procedures dendritic cells could be generated from thawed populations of PBMC. Although not investigated systematically, it is preferred to freeze the starting population of PBMC or immature dendritic cells on day 7 of culture [i.e.], the day of transfer into CM-containing medium rather than mature cells on day 10/11 of culture.

55 [0130] The criteria used initially to define fully mature dendritic cells included: morphology, lack of adherence to plastic, and select surface markers. Dendritic cells grown for six days in GM-CSF and IL-4, i.e., immature dendritic cells did not express CD83 but did express CD115 [CSF-1 receptor/M-CSF receptor]. When they were replated without cytokines [and without CM] they adhered to the bottom of the culture wells. After a three day culture in the presence of

CM they became CD83+/CD115- and did not become adherent upon withdrawal of cytokines. They also maintained their pronounced cytoplasmic, motile "veils". These results are consistent with the production of fully and stably mature dendritic cells. Only SACS, conditioned media from SACS-stimulated adherent PBMC, and conditioned media from monocytes stimulated by adherence on Ig were able to make dendritic cells reach full maturation. When we tried to replace CM with cytokines we observed no effects or some increase in T cell stimulatory capacity [with IL-1 $\beta$ , and/or TNF- $\alpha$  or IL-15]. Full maturation was not achieved, however. In as shown in Example 1, neutralizing antibodies to IL-1 $\alpha$  and  $\beta$ , IL-6, TNF- $\alpha$ , IL-12, alone or in combination, do not block the activity of the monocyte CM. CM may contain either a unique cocktail of cytokines that is difficult or impossible to reproduce experimentally or new cytokines.

### EXAMPLE 3

#### Clinical Use of Dendritic Cells Primed With Influenza or HIV Antigens

[0131] In this Example, dendritic cells prepared as described in Example 1 are primed with peptides that are presented on MHC class I molecules to prime CD8+ T cells. We have used the immunodominant influenza matrix peptide, GILGFVFTL, that is recognized by CTLs from HLA-2.1 individuals, and we will in addition use HIV-1 peptides [pol ILKEPVHC-V; gag SLYNTVATL] that are recognized by CTLs from HIV-1 infected individuals. The efficacy of the peptide pulse can be monitored by applying an aliquot of the DCs to autologous T cells in culture and measuring the killer cell response.

[0132] Most humans are primed to influenza antigens, so that the recall CTL response to this virus provides an optimal and internal control to determine if autologous DCs, pulsed ex vivo with viral antigens, can boost the CTL response in vivo.

[0133] In this protocol, HIV-1 infected individuals are treated with CD4+ T cell counts of 300-400/uL. These individuals have low numbers of HIV-1 specific, killer cell precursors. Asymptomatic, HIV-1 infected, adult volunteers of both sexes will be recruited. The donors must be HLA-A2.1 positive, as will be determined by initial typing with a monoclonal to A2.1. Donors who have relatively low and high recall responses to influenza will be included. The therapeutic goal is to increase the numbers of these killers in order to reduce the cell-cell spread of HIV-1. The killer cell response to influenza is an internal control, since it should be intact in HIV-1 infected patients.

[0134] Pairs of HIV-1 peptides are used so that the response to dendritic cells administered by two routes, intradermal [i.d.] and subcutaneous [s.c.] can be compared in the same patient. The influenza peptide will serve as the common denominator for each route. The peptides are made according to Good Laboratory Practices [GLP] at Sloan Kettering Institute, where peptides have been synthesized previously for administration in humans.

#### The Clinical Protocol is as follows:

##### [0135]

- a. Take a 50-60 ml blood sample in heparin by venipuncture.
- b. Isolate the mononuclear cells on Ficoll Hypaque, and save the autologous plasma.
- c. Separate the mononuclear cells into T cell enriched and T cell depleted fractions by sheep erythrocyte rosetting, or by depleting lymphocytes with GMP-grade DYNAL beads coated with monoclonal antibodies to B and T cells. The T cells are frozen for tissue culture tests for antigen presentation to cytolytic T lymphocytes [CTLs, below].
- d. Culture a fraction of the mononuclear cells on culture plates that have been coated with human Ig [Sandoz] in an RPMI-1640 culture medium supplemented with antibiotics [penicillin and streptomycin]. This stimulates the cells to form a conditioned medium that fosters DC maturation.
- e. Culture the bulk of the T-depleted mononuclear cells for 6 days in RPMI-1640 supplemented with antibiotics, 1% autologous plasma, 1000 U/ml rHuGM-CSF, and 1000 U/ml rHuIL-4. GMP grade cytokines are provided by Schering-Plough.
- f. After 6 days, transfer the cells to fresh medium supplemented with 30-50% v/v autologous monocyte conditioned medium plus 1  $\mu$ M of influenza matrix peptide, GILGFVFTL. One aliquot of the DCs is pulsed with the HIV-1 gag peptide and the other with the polymerase peptide. At day 11, when the DCs have acquired their typical stellate shapes, the DCs are harvested, washed, counted, and readied for injection by s.c. and i.d. routes. Prior to the injection, the patients are typed to be sure that they have killer cell precursors specific for the above peptides.
- g. The DCs are injected in the forearm. On one side the cells are given in a total of 0.5ml in 3 intradermal sites, while on the other side, the cells are given in 0.5 ml in 3 subcutaneous sites. The purpose of the two routes is to compare the efficacy of boosting to the influenza matrix peptide.
- h. 1 week and 1 month after injection, 25 ml of blood is taken to evaluate the induction of immune responses. T cell responsiveness to the peptide is measured by antigen-induced proliferation and release of IFN-gamma in culture.

The results are compared to preinjection values which are obtained 0 and 1 month prior to injection. The first injection utilizes DCs that are not pulsed with peptide. T cell responsiveness to peptide will be measured by enumerating CTL precursor frequencies [CTLp] for the peptide-coated, HLA-A2.1 "T2" cell line.

i. If priming is not detected at 1 month, a second dose of antigen pulsed DCs is administered. If priming is detected at 1 month, the immune status is reexamined at 3 months and 6 months to determine the longevity of immune memory.

#### Example 4

#### Development of Minimal Dendritic Cell Maturation Factor

[0136] MCM was analyzed for candidate factors including  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$ , which are of particular interest since they induce the maturation of GM-CSF/IL-4 primed dendritic cells that are generated in medium containing FCS (Sallusto, 1994; Sallusto, 1995; Zhou, 1996). Dendritic cells generated in MCM versus specific cytokine combinations were compared with respect to features associated with terminal maturation, including phenotypic stability and potent T cell stimulatory capacity.

#### Cytokine concentrations in MCM

[0137] MCM substantially increases the yield [3-10 fold], enrichment [5-15 fold], and immunostimulatory [5-15 fold] capacity of dendritic cells generated from GM-CSF/IL-4 treated precursor cells in blood (Bender, 1996; Romani, 1996). The MCM-generated cells are phenotypically and functionally stable in that they retain dendritic cell features for several days when cultured in the absence of cytokines, characteristics of full maturation (Bender, 1996; Romani, 1996). We therefore analyzed several MCM preparations for the presence of cytokines that could be candidates for maturation factors, such as  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , IL-6 and  $\text{IFN}\alpha$ . Using commercially available ELISA kits, all four cytokines were detected, but in widely varying concentrations [Table 9]. In some instances the concentrations were high [up to 1  $\mu\text{g/ml}$  for IL-6]. Notably, each of the MCM preparations analyzed was active in inducing dendritic cell maturation at a concentration of 50% v/v.

TABLE 9

Concentrations of cytokines in MCM [ng/ml]				
	$\text{TNF}\alpha$	$\text{IL-1}\beta$	IL-6	$\text{IFN}\alpha$
Range	9 - 182	1.5 - 92	6 - 1,000	.02 - .1
Mean +/- S.D.	41 $\pm$ 58	27.7 $\pm$ 37	584.5 $\pm$ 420	.04 $\pm$ .02
n =	7	5	4	17

Table 9: Analysis of cytokines in MCM

[0138] Several MCM preparations were evaluated for the presence of  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , IL-6, and  $\text{IFN}\alpha$  using standard ELISA kits. The range of concentrations and the mean +/- S.D. [ng/ml] for 4-17 samples are given.

#### Contribution of $\text{TNF}\alpha$ in the maturation of dendritic cells

[0139]  $\text{TNF}\alpha$ , in conjunction with other cytokines, is critical for the development of dendritic cells from CD34+ progenitor cells in cord blood and bone marrow (Szabolcs, 1995; Young, 1995; Santiago-Schwarz, 1992; Reid, 1992; Caux, 1996). It also mediates the maturation of GM-CSF/IL-4 primed blood progenitor cells that are cultured in medium with FCS (Sallusto, 1994; Sallusto, 1995; Zhou, 1996). Since substantial quantities of  $\text{TNF}\alpha$  are present in MCM, we evaluated its role in the maturation of dendritic cells generated in medium containing 1% plasma.  $\text{TNF}\alpha$  [50 ng/ml] was added to GM-CSF/IL-4 treated ER- cells every other day on days 0, 2, 4, 6 and 8. After 11 days of culture, the cells were collected and assayed for immunostimulatory activity in the allo MLR and for CD83 expression [Fig. 12]. Residual cells were washed, returned to culture in the absence of additional cytokines and reanalyzed 3 days later [day 14], to determine whether the cells had irreversibly matured. Compared to GM-CSF/IL-4 treatment alone,  $\text{TNF}\alpha$  did generate cells that expressed CD83 [Fig. 12, upper panel], moderate levels of MHC molecules, adhesins and costimulators [e.g. CD80 and CD86, not shown] and that were potent stimulators of allogeneic T cells as previously described (Zhou, 1996).

However, they were less immunostimulatory than MCM-treated cells, and 60% fewer cells were obtained [ $2.1 \times 10^6$  vs.  $5 \times 10^6$  for MCM treated cells on day 11].

**[0140]** By day 14, a significant proportion of  $\text{TNF}\alpha$ -treated cells readhered to plastic or died, as reflected in the reduced yields [ $0.6 \times 10^6$ ]. Furthermore, there was substantial down-regulation of CD83 [Fig. 1, lower panel] and MHC molecules [not shown], and re-expression of CD14 [data not shown].  $\text{TNF}\alpha$ -treated cells also failed to express the activation marker CD25. Similar results were obtained when different concentrations of  $\text{TNF}\alpha$  [10-100 ng/ml] were used or if it was added only on day 7.

**[0141]** In contrast to  $\text{TNF}\alpha$ , MCM treatment induced stable CD83 expression, high levels of MHC and costimulator molecules, and recovery of substantially higher cell numbers [ $3.7 \times 10^6$ , Fig. 1, lower panel]. Cells cultured in the absence of either MCM or  $\text{TNF}\alpha$  had the least immunostimulatory activity, generated the fewest number of cells [ $0.5 \times 10^6$ ], and had low to no CD83 expression. Therefore,  $\text{TNF}\alpha$  alone does not account for the terminal maturational effects induced by MCM.

#### Minimal dendritic cell maturation factor

**[0142]** We next ascertained whether a combination of cytokines could substitute for MCM.  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6 and IFN $\alpha$  were added in combination to GM-CSF/IL-4 treated ER-cells on day 7. After 11-14 days in culture the cells were enumerated and analyzed for stimulatory capacity [Figure 14] and CD83 expression [Figure 15]. In all experiments cells were washed on days 7 and 11 prior to analysis, to remove residual cytokines. In Figure 14 C and D, the cytokines were added back in concentrations equivalent to those measured by ELISA in the corresponding MCM. The cytokine combination enhanced the immunostimulatory function of GM-CSF/IL-4 treated cells in the allo MLR and in some cases was similar to that seen with MCM [Figure 14 B]. However, in other experiments [Figure 14 A and D], the cytokines did not fully substitute for MCM. This combination of cytokines is nevertheless effective since cells upregulated and retained CD83 expression for at least 3 days after cytokine removal [Figure 15].

**[0143]** To identify which cytokines comprised an effective combination, all possible mixtures were tested. MCM consistently generated the greatest stimulatory activity on a per cell basis [above and data not shown], highest yields, and CD83 expression [Figure 16] and was therefore considered the standard against which other culture conditions should be assessed. For example, in the absence of MCM, on average only 42% of the maximal cell yield was obtained [of which few are actually mature dendritic cells]. Cytokine cocktails increased the yield up to or as much as 69%. To evaluate stable CD83 expression, cells treated with various combinations of cytokines were washed on day 11 and recultured for 3 more days in the absence of additional supplements. CD83 could be induced on variable numbers of cells by several combinations of cytokines, but not by individual cytokines. MCM generated the highest number of CD83+ cells. Collectively, the data suggest that the effects of MCM are complex and probably due to the combined effect of several cytokines, including those evaluated here.

**[0144]**  $\text{TNF}\alpha$  was a prime candidate for a dendritic cell maturation factor, since it matures dendritic cells grown in GM-CSF/IL-4 in the presence of FCS (Sallusto, 1994; Sallusto, 1995; Zhou, 1996). However, our results differ in two significant respects from these studies. First, we found that  $\text{TNF}\alpha$ 's effects on dendritic cell development were transient when 1% plasma was used in the place of FCS. Removal of the cytokine resulted in a substantial decrease in cell number. The cells acquired macrophage-like properties [adherence to plastic and CD14 expression] and CD83 expression was down-regulated. This fact was not previously appreciated since dendritic cells are generally studied without reculturing them in the absence of cytokines (Sallusto, 1994; Sallusto, 1995; Zhou, 1996). Second, dendritic cells generated in GM-CSF/IL-4 and  $\text{TNF}\alpha$  in FCS express CD1a and low levels of CD25 (Zhou, 1996). The loss of CD1a and upregulation of CD25 are associated with the terminal maturation of both blood and skin derived dendritic cells, which are CD83+, CD1a- and CD25+ (Bender, 1996; Romani, 1996).

**[0145]** Other stimuli aside from  $\text{TNF}\alpha$  that have been reported to induce dendritic cell maturation from GM-CSF/IL-4 primed progenitors include IL-1, LPS and CD40-L (Sallusto, 1994; Sallusto, 1995). However, as with  $\text{TNF}\alpha$ , these studies used cells generated in FCS, which could provide additional growth inducing factors. We have studied IL-1 $\beta$  and Pansorbin [SAC] in our culture system. IL-1 $\beta$  alone was significantly less effective than MCM. Conditioned medium that is generated by stimulating monocytes with Pansorbin for 24 hours, can substitute for MCM, probably by inducing the production of relevant cytokines (Bender, 1996). LPS contaminants are unlikely to explain the effects of MCM since we did not detect physiologically significant levels in several preparations by the Limulus amoebocyte lysate assay [data not shown].

**[0146]** The responsible factor[s] in MCM probably include some combination of  $\text{TNF}\alpha$ , IL-1 $\beta$ , IL-6 and IFN $\alpha$ , since these could substitute for MCM. Previous attempts to neutralize  $\text{TNF}\alpha$ , IL-1 $\beta$  and IL-6 in MCM preparations with antibodies were unsuccessful (Bender, 1996). However, it is equally likely that other components, not yet defined, are also critical to obtain 100% of the activity of the conditioned medium. For example, MCM contains the chemokines MIP-1 $\alpha$  and Rantes [data not shown]. IL-12, a factor that augments dendritic cell dependent T cell stimulatory capacity (Koch, 1996; Bhardwaj, 1996; Cella, 1996) is also present in MCM, but neutralization of this factor with mAbs does not affect

MCM activity (Bender, 1996). Possibly a new factor, or even production of factors by the maturing dendritic cells themselves are responsible for optimal development. TGF- $\beta$ 1 is one candidate. This cytokine appears to be essential for Langerhans cell development or epidermal localization (Borkowski, 1996). The complexity and diversity of MCM makes it difficult to identify every relevant component. Nevertheless, the significance of the cell maturation factor in MCM is underscored by our earlier observations that MCM is vital for the maturation of a subset of circulating blood dendritic cells (O'Doherty, 1993; O'Doherty, 1994). This effect however, can be nearly duplicated by combination of individual cytokines as shown herein.

**[0147]** Evidence for a physiologic counterpart for MCM comes from studies showing that dendritic cells are rapidly induced to migrate into tissues [lung (McWilliams, 1994), synovial fluid (Zvaifler, 1985; Bhardwaj, 1988; Thomas, 1994)], and from organs [gut, heart, kidney, skin (MacPherson, 1995; Roake, 1995; Austyn, 1994; Weinlich, 1989)] into lymph or lymphoid tissue in response to certain stimuli, e.g. LPS, infection, chronic inflammation. Migration parallels maturation as determined by the downregulation of antigen processing capacity and upregulation of T cell stimulatory function (De Smedt, 1996).

**[0148]** While we have hereinbefore described a number of embodiments of this invention, it is apparent that the basic constructions can be altered to provide other embodiments which utilize the methods and compositions of the invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have presented hereinbefore by way of example.

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[0226] All of the foregoing are incorporated herein by reference.

## Claims

1. An *in vitro* method of producing mature dendritic cells from immature dendritic cells derived from pluripotent cells having the potential of expressing either macrophage or dendritic cell characteristics, said method comprising contacting the immature dendritic cells with a dendritic cell maturation factor wherein the dendritic cell maturation factor comprises IFN $\alpha$ .

2. The method of claim 1, wherein the dendritic cell maturation factor further comprises at least one other cytokine selected from the group consisting of IL-1 $\beta$ , IL-6 and TNF $\alpha$ .

3. The method of claim 2, wherein the dendritic cell maturation factor comprises about 20 to about 200 ng/ml TNF $\alpha$ ; about 20 to about 100 ng/ml IL-1 $\beta$ ; about 20 to about 1000 ng/ml IL-6; and about 0.02 to about 0.08 ng/ml IFN $\alpha$ .

4. The method of claim 2, wherein the dendritic cell maturation factor comprises about 20 ng/ml TNF $\alpha$ ; about 20 ng/ml IL-1 $\beta$ ; about 20 ng/ml IL-6; and about 0.02 ng/ml IFN $\alpha$ .

5. An *in vitro* method of producing irreversibly mature dendritic cells, said method comprising contacting pluripotent cells with at least one first factor to produce an immature dendritic cell and contacting the immature dendritic cell with a dendritic cell maturation factor wherein the dendritic cell maturation factor comprises IFN $\alpha$  to produce irreversibly mature dendritic cells.

6. The method of claim 5, wherein the first factor comprises at least one cytokine selected from the group consisting of GM-CSF and IL-4.

7. The method of claim 5, wherein the dendritic cell maturation factor further comprises at least one other cytokine selected from the group consisting of IL-1 $\beta$ , IL-6 and TNF $\alpha$ .

8. The method of claim 5, wherein the dendritic cell maturation factor comprises about 20 to about 200 ng/ml TNF $\alpha$ ; about 20 to about 100 ng/ml IL-1 $\beta$ ; about 20 to about 1000 ng/ml IL-6; and about 0.02 to about 0.08 ng/ml IFN $\alpha$ .

9. The method of claim 5, wherein the dendritic cell maturation factor comprises about 20 ng/ml TNF $\alpha$ ; about 20 ng/ml IL-1 $\beta$ ; about 20 ng/ml IL-6; and about 0.02 ng/ml IFN $\alpha$ .

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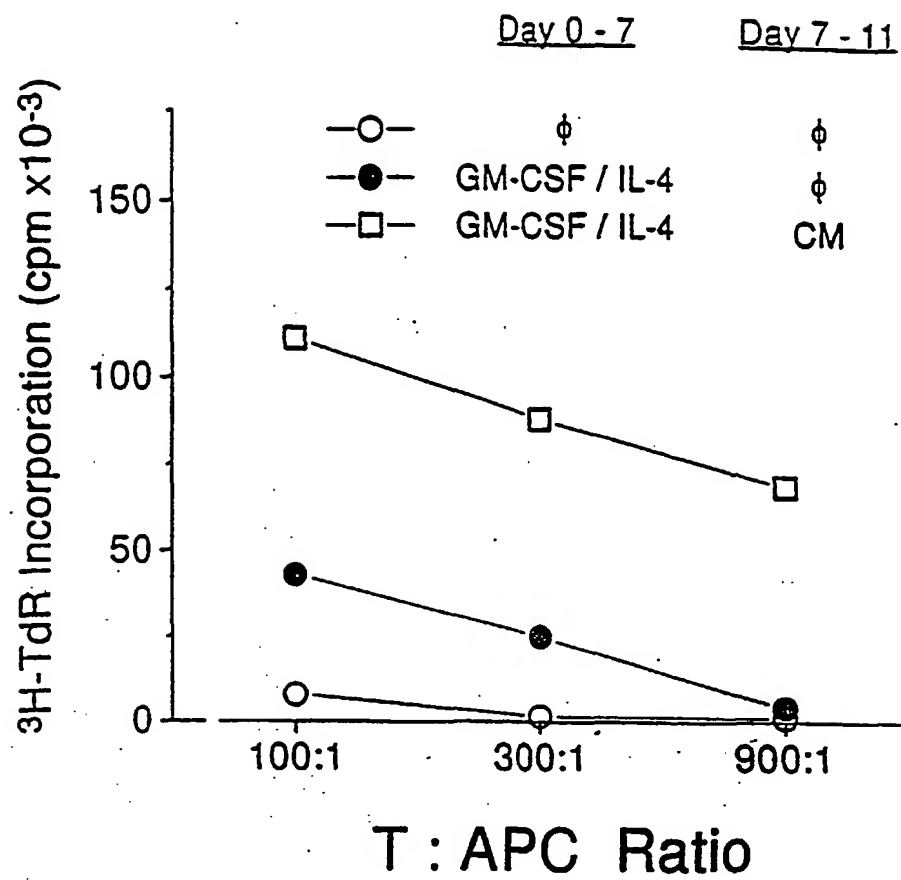


FIG. 1

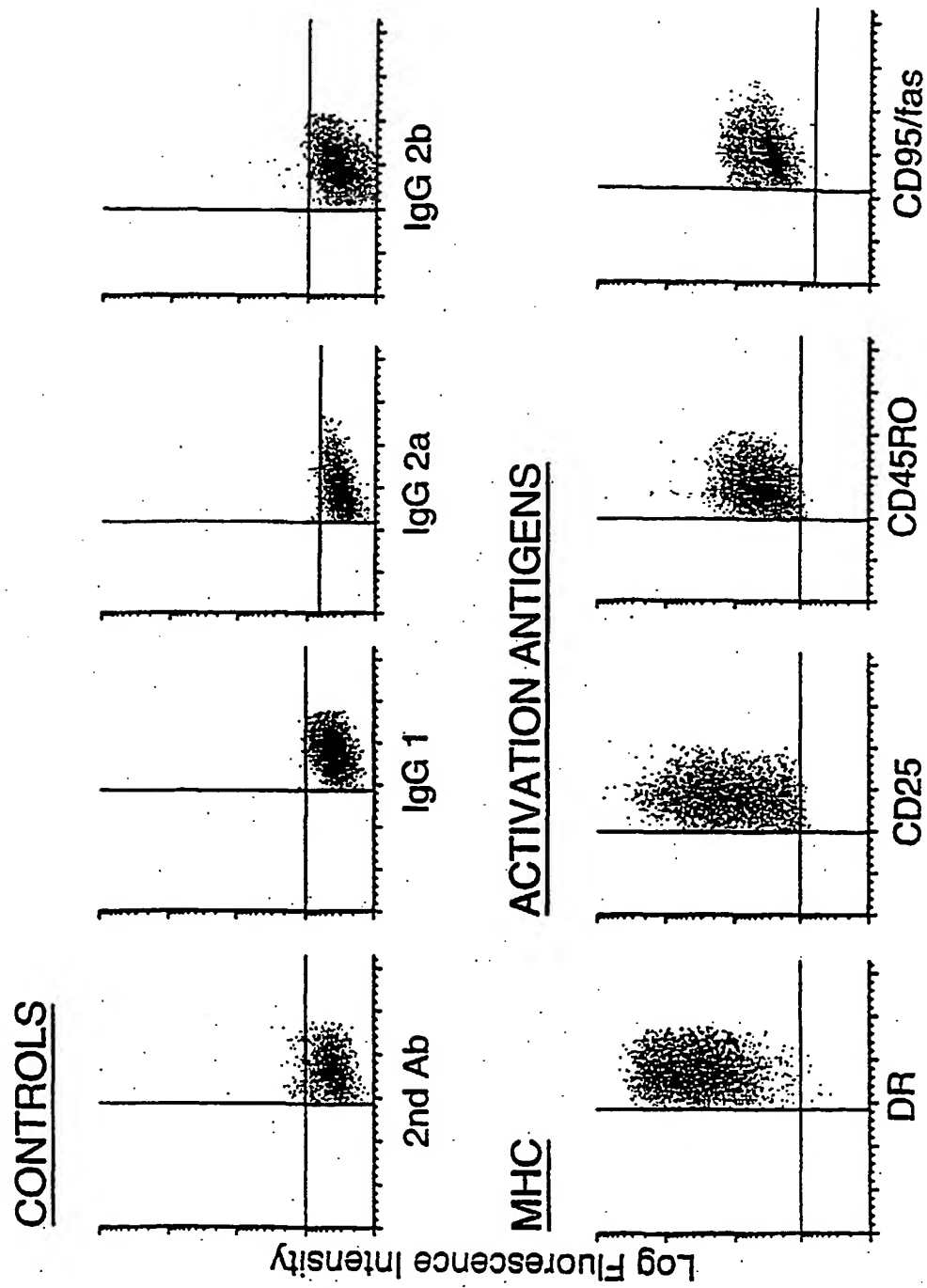


FIG. 2

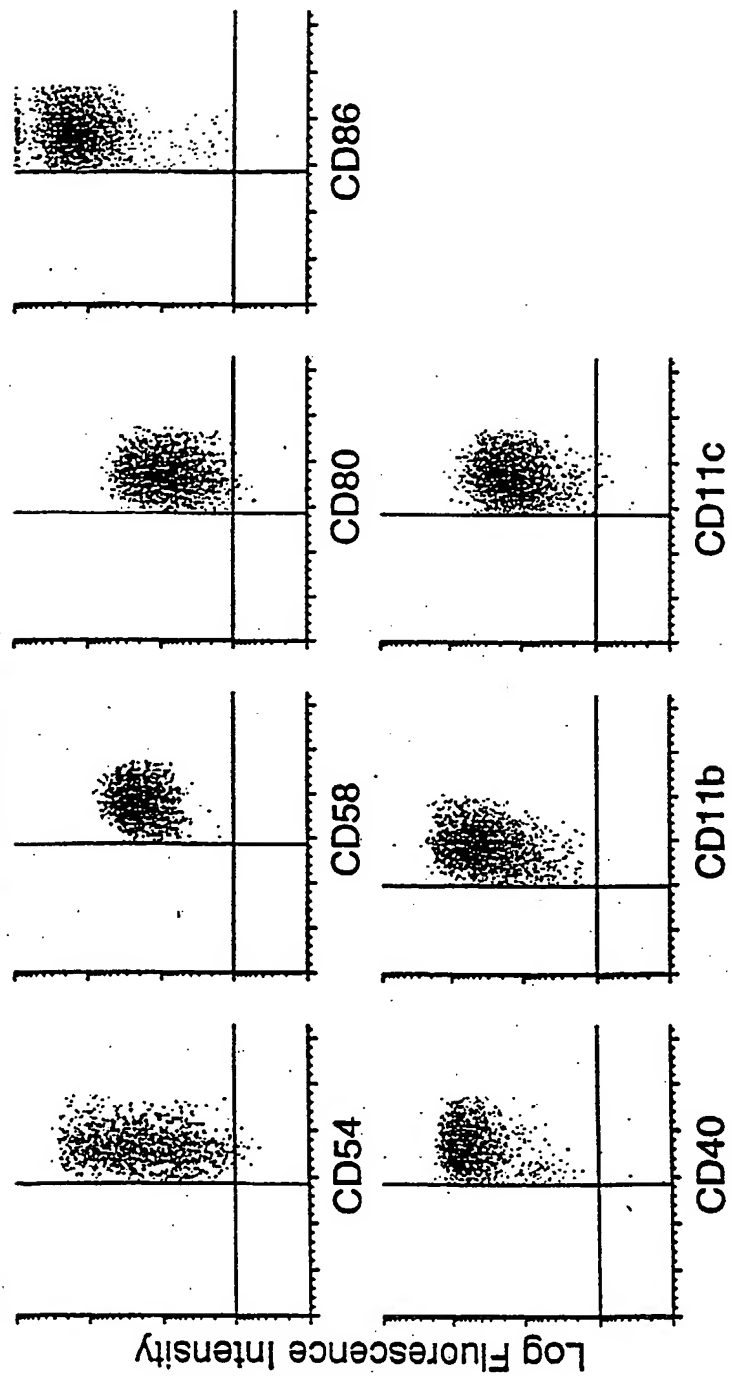
COSTIMULATORS / ADHESINS

FIG. 2

LINEAGE RESTRICTED ANTIGENS

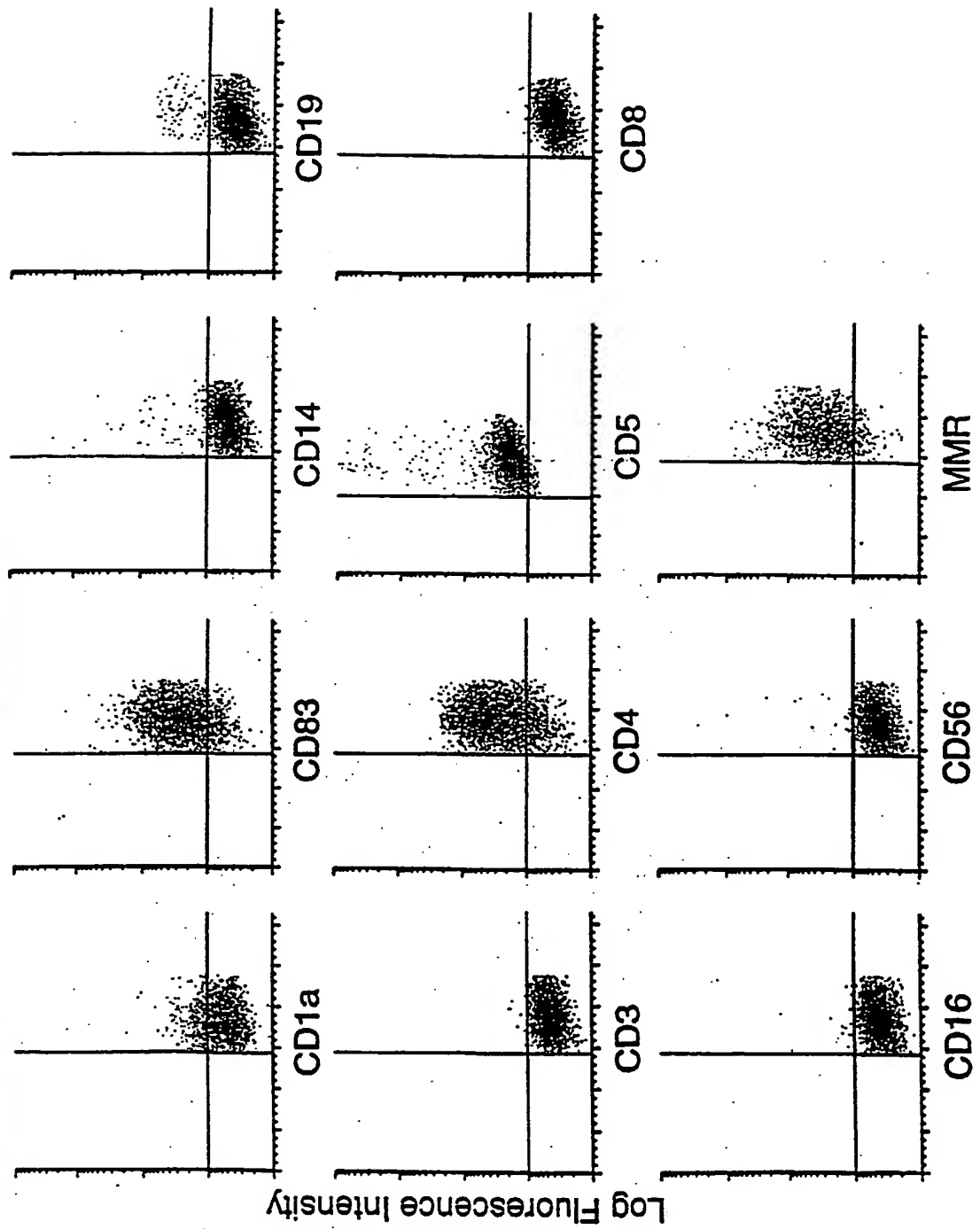
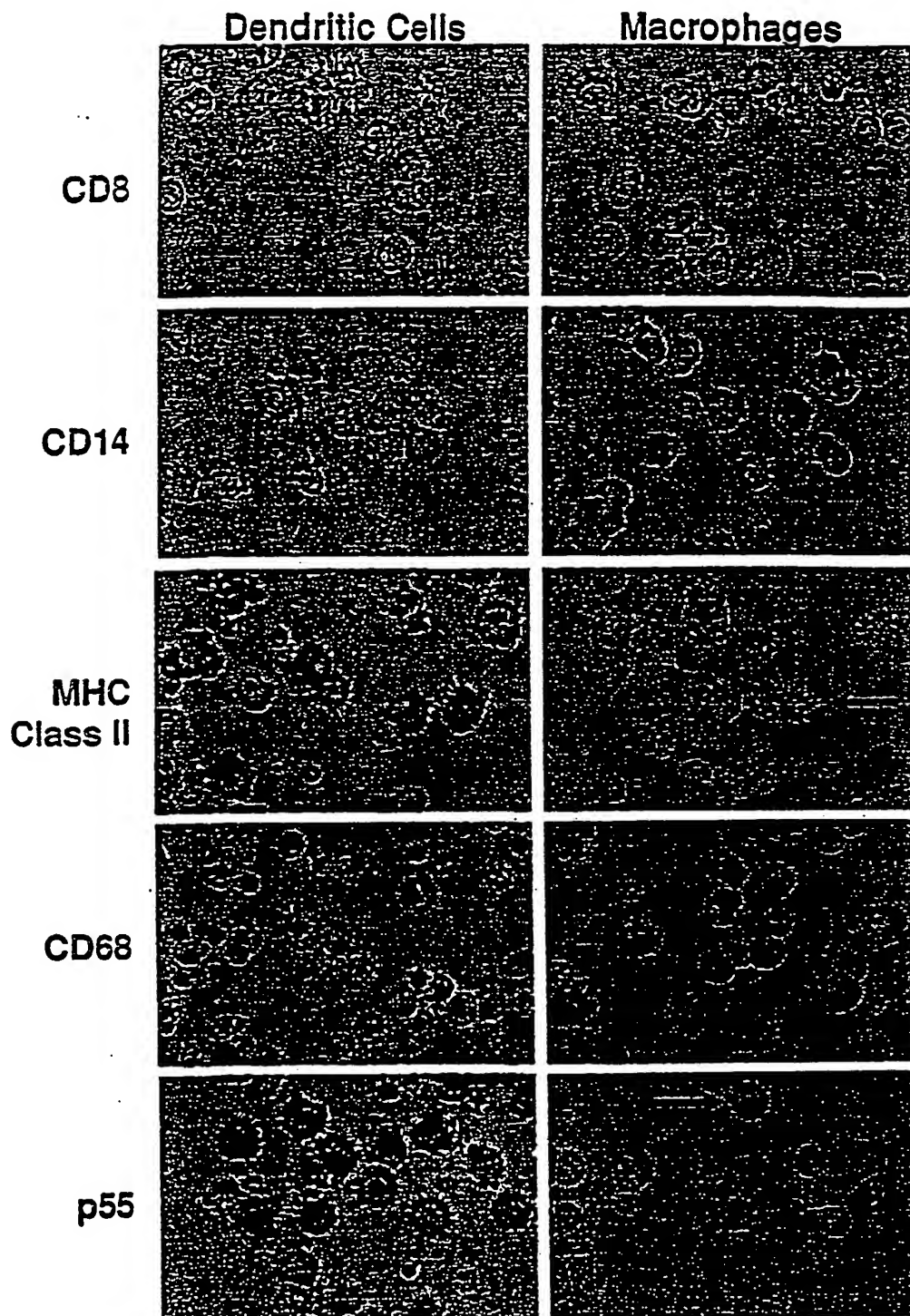


FIG. 2

*FIG. 3*

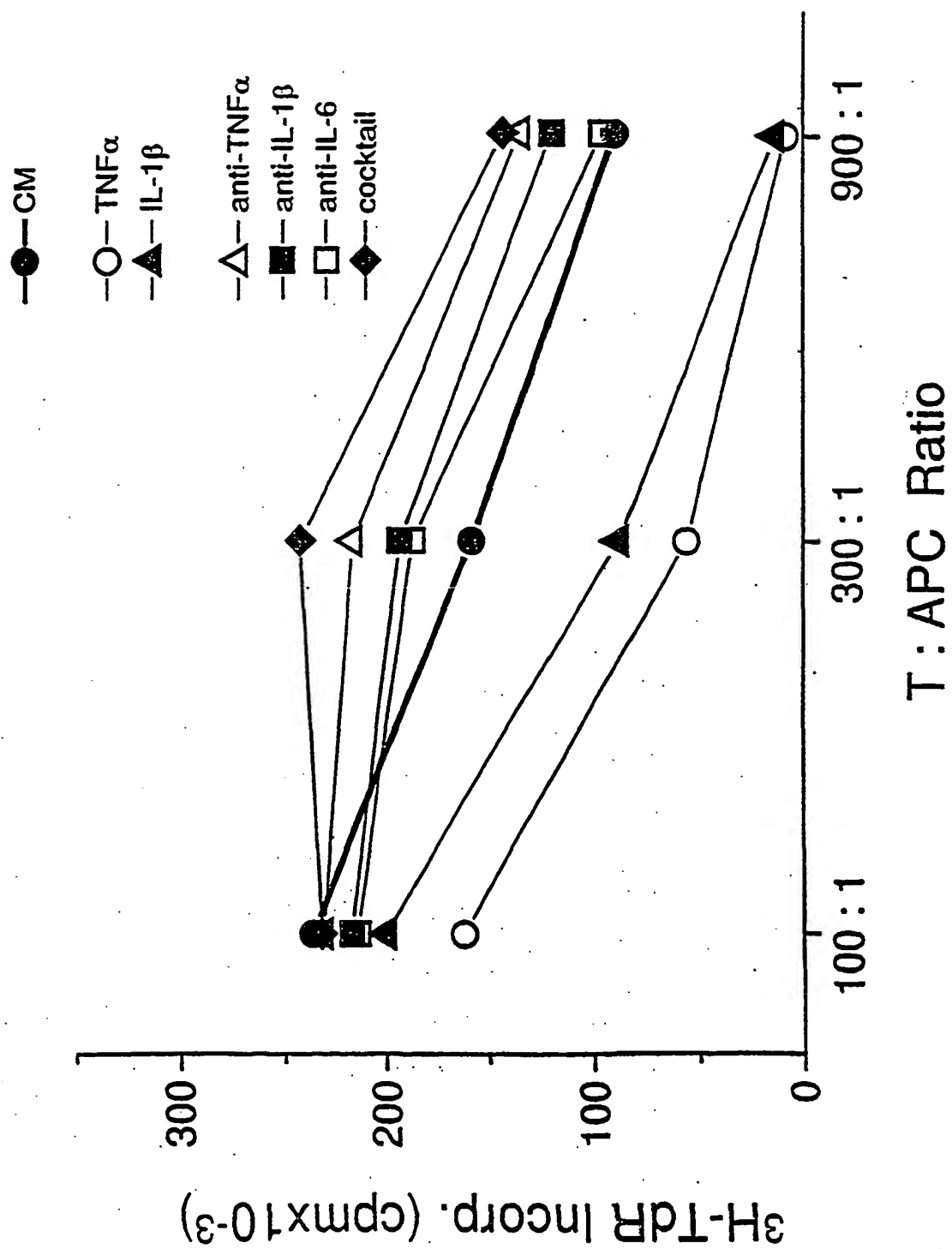


FIG. 4

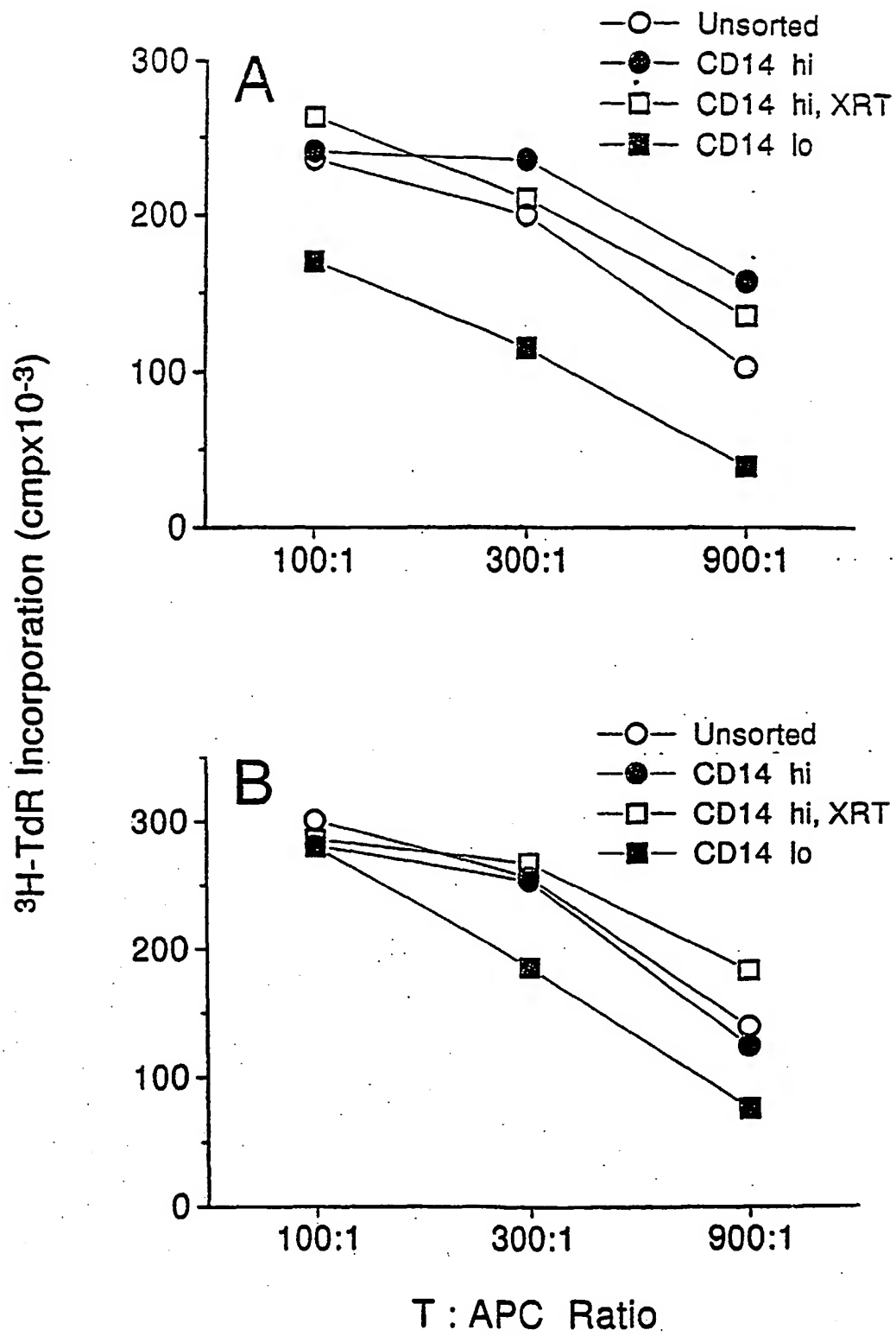
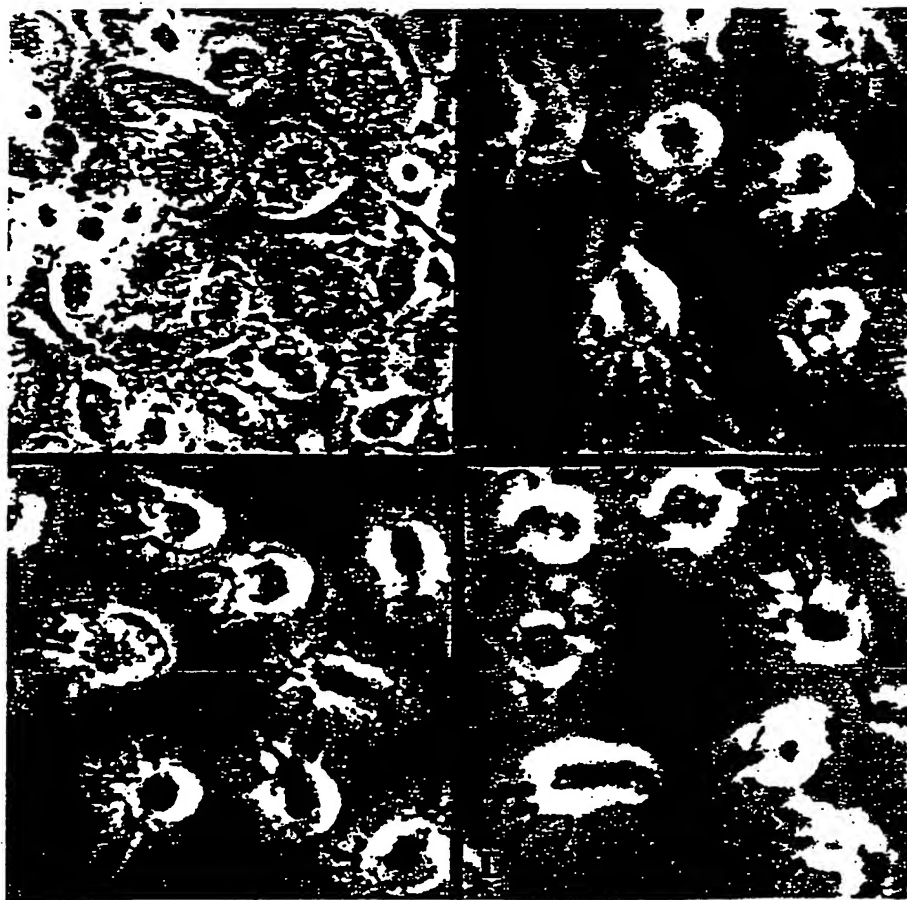


FIG. 5



*FIG. 6*

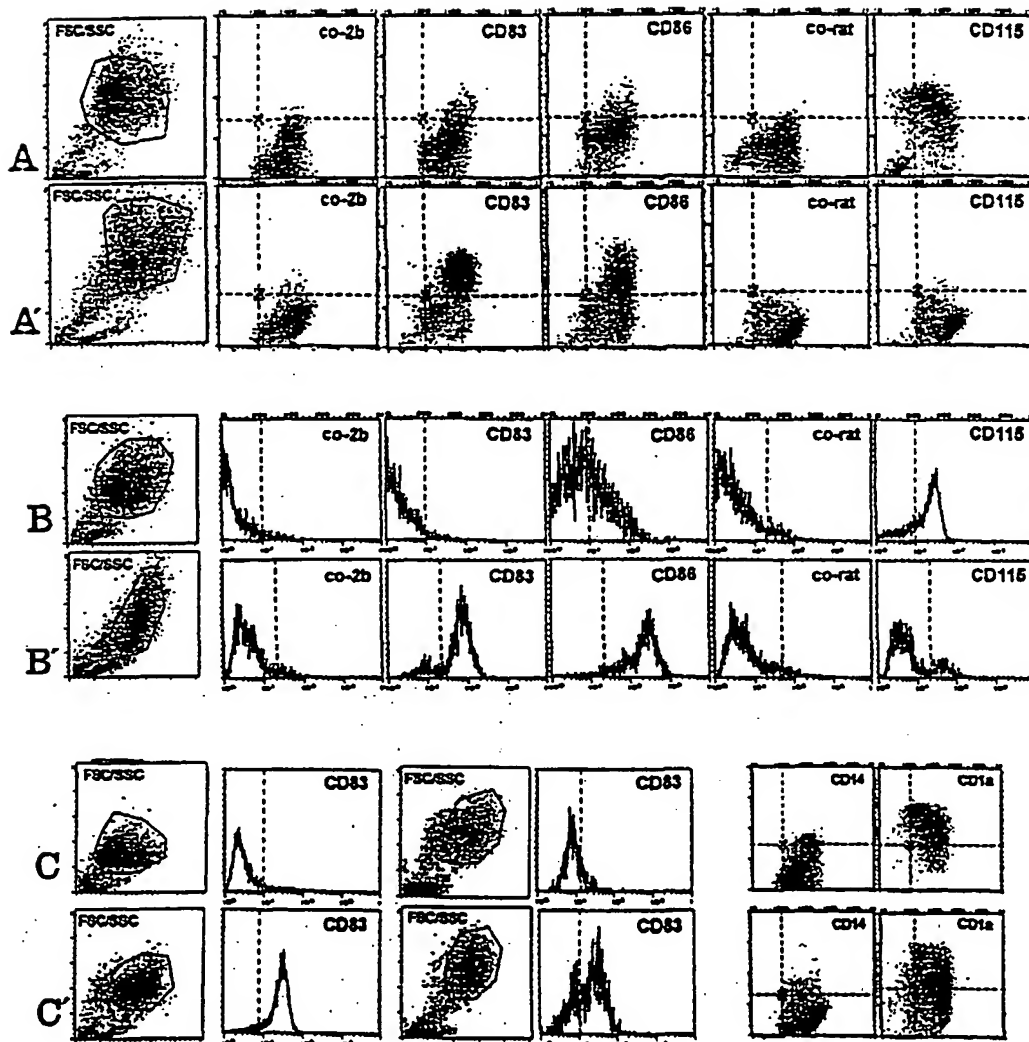


FIG. 7

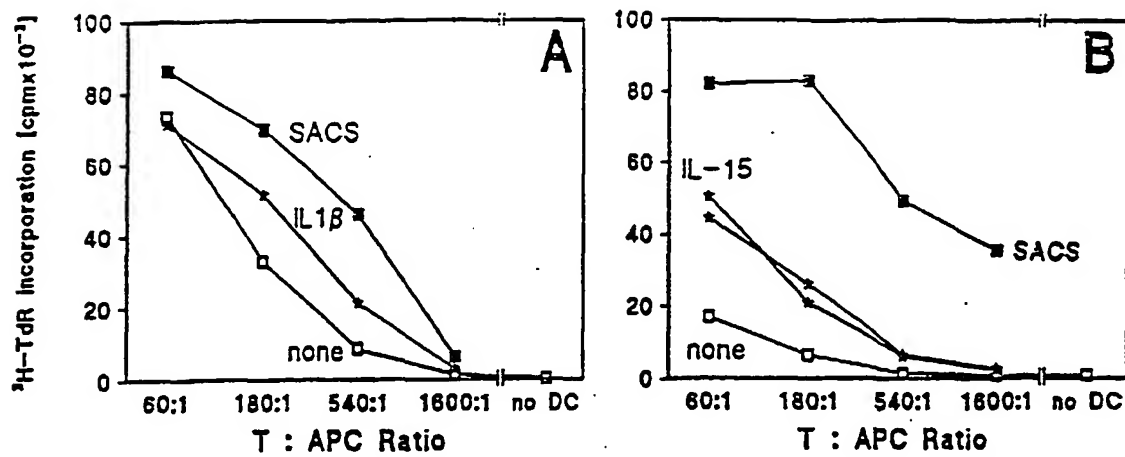
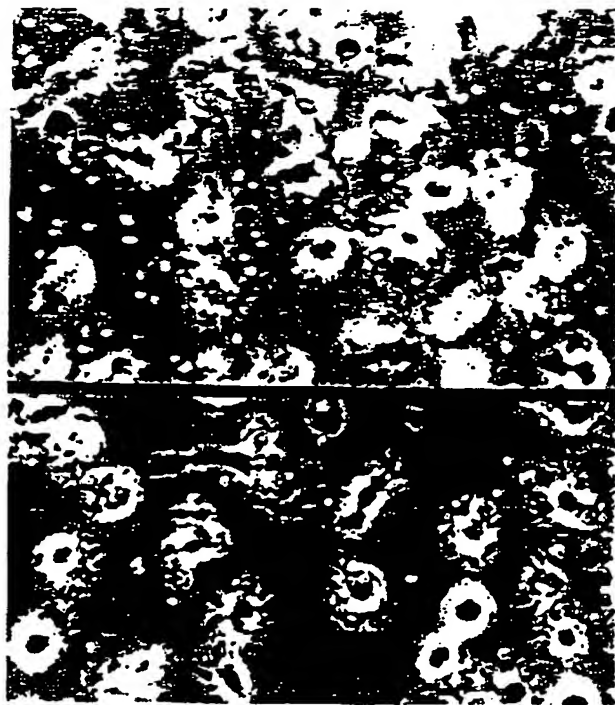


FIG. 8



*FIG. 9*



*FIG. 10*

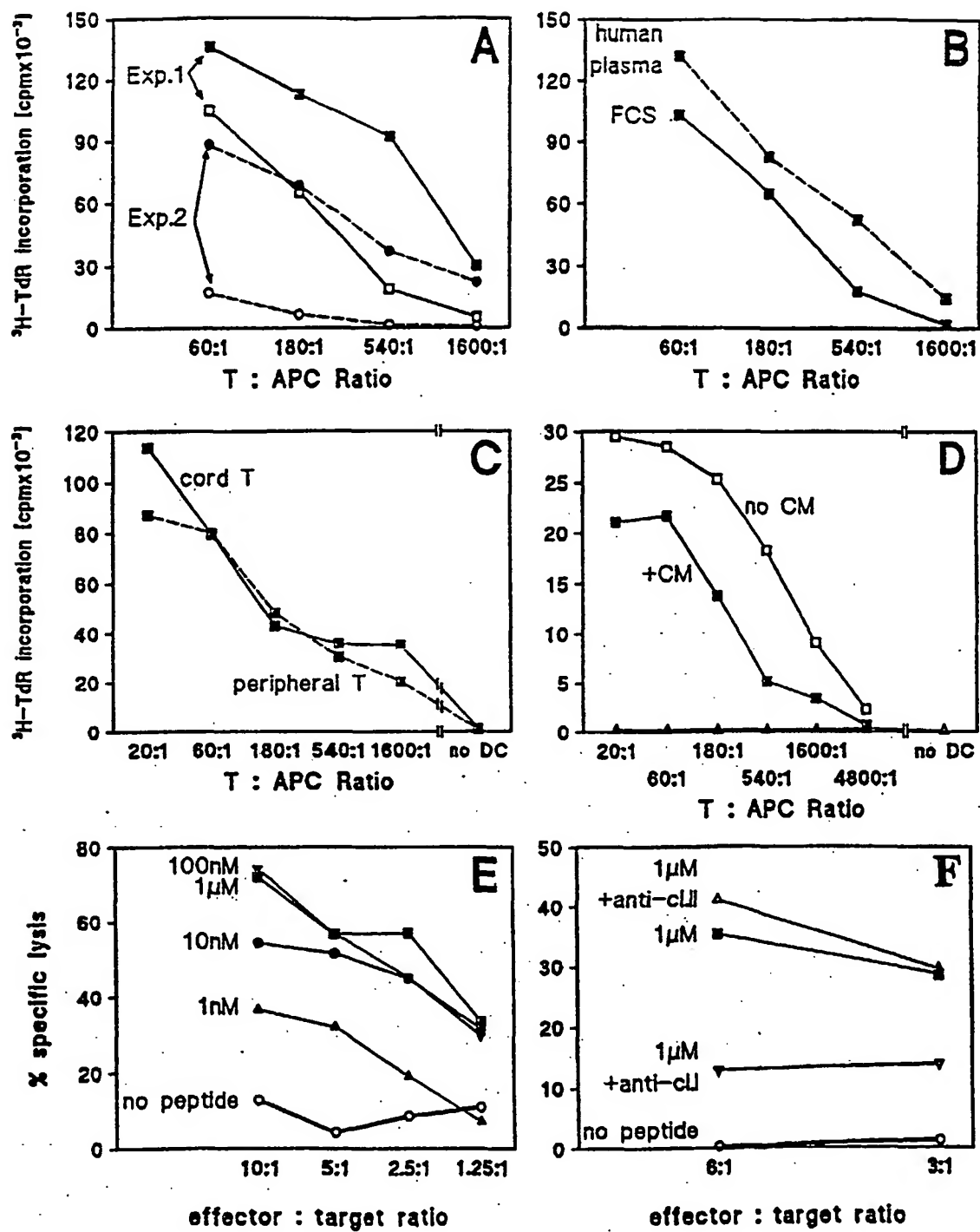


FIG. 11

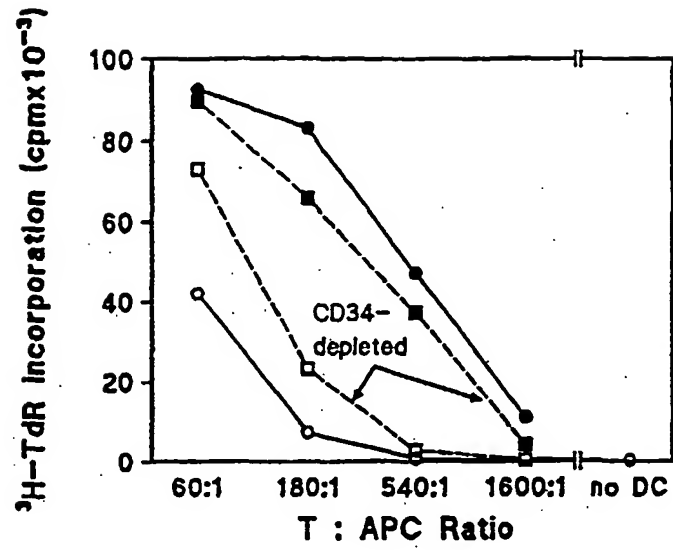


FIG. 12

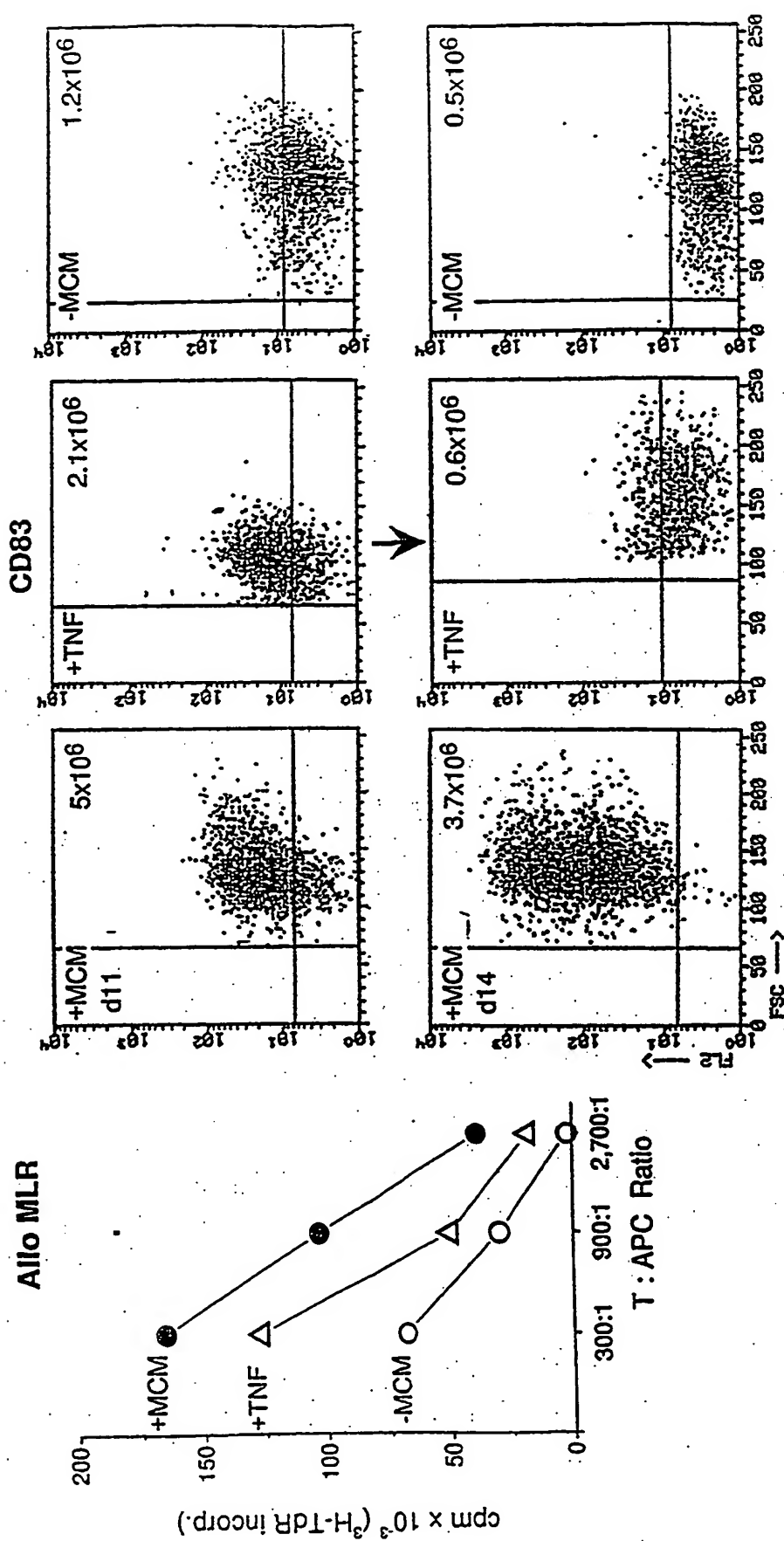


FIG. 13

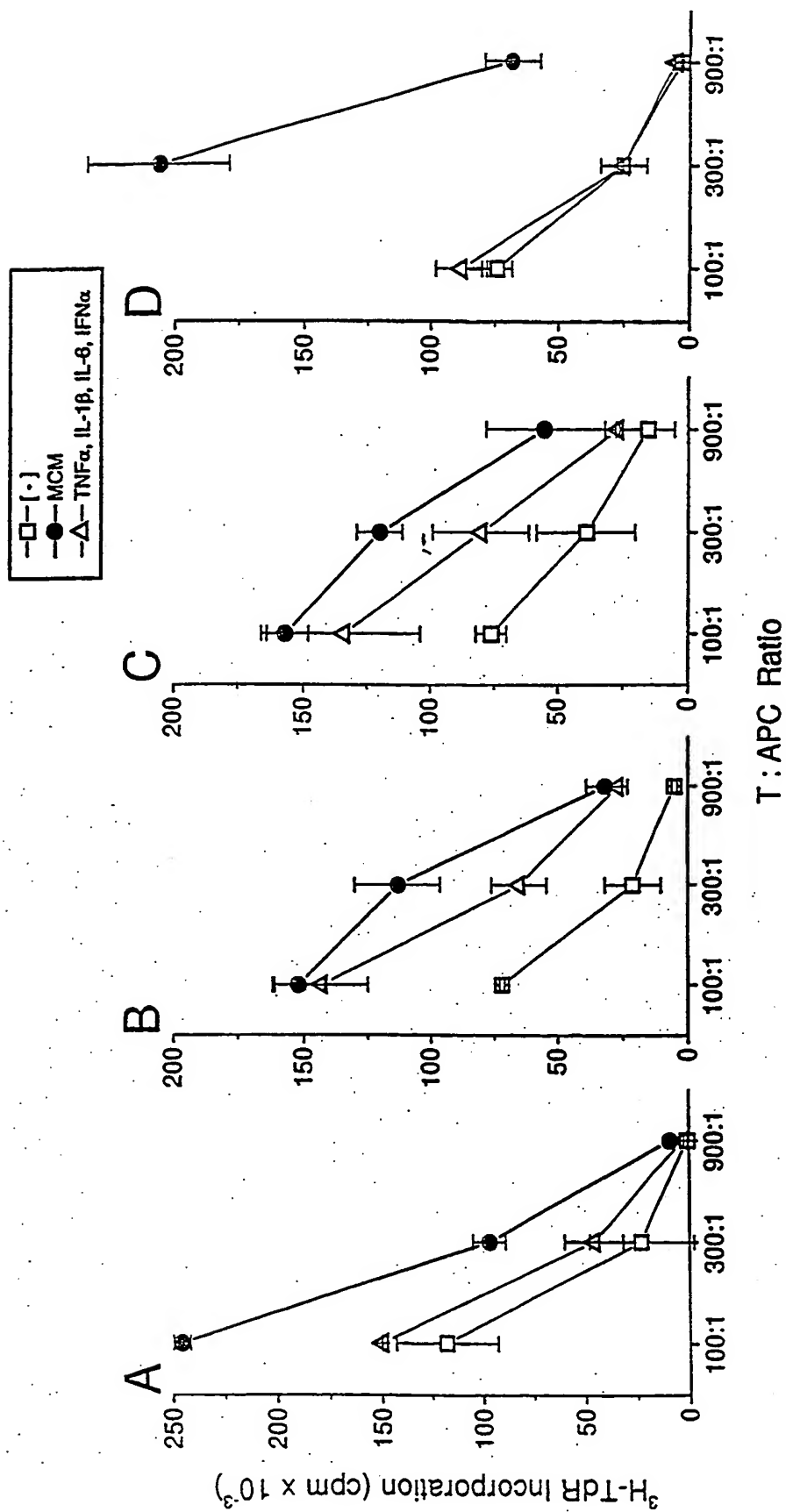
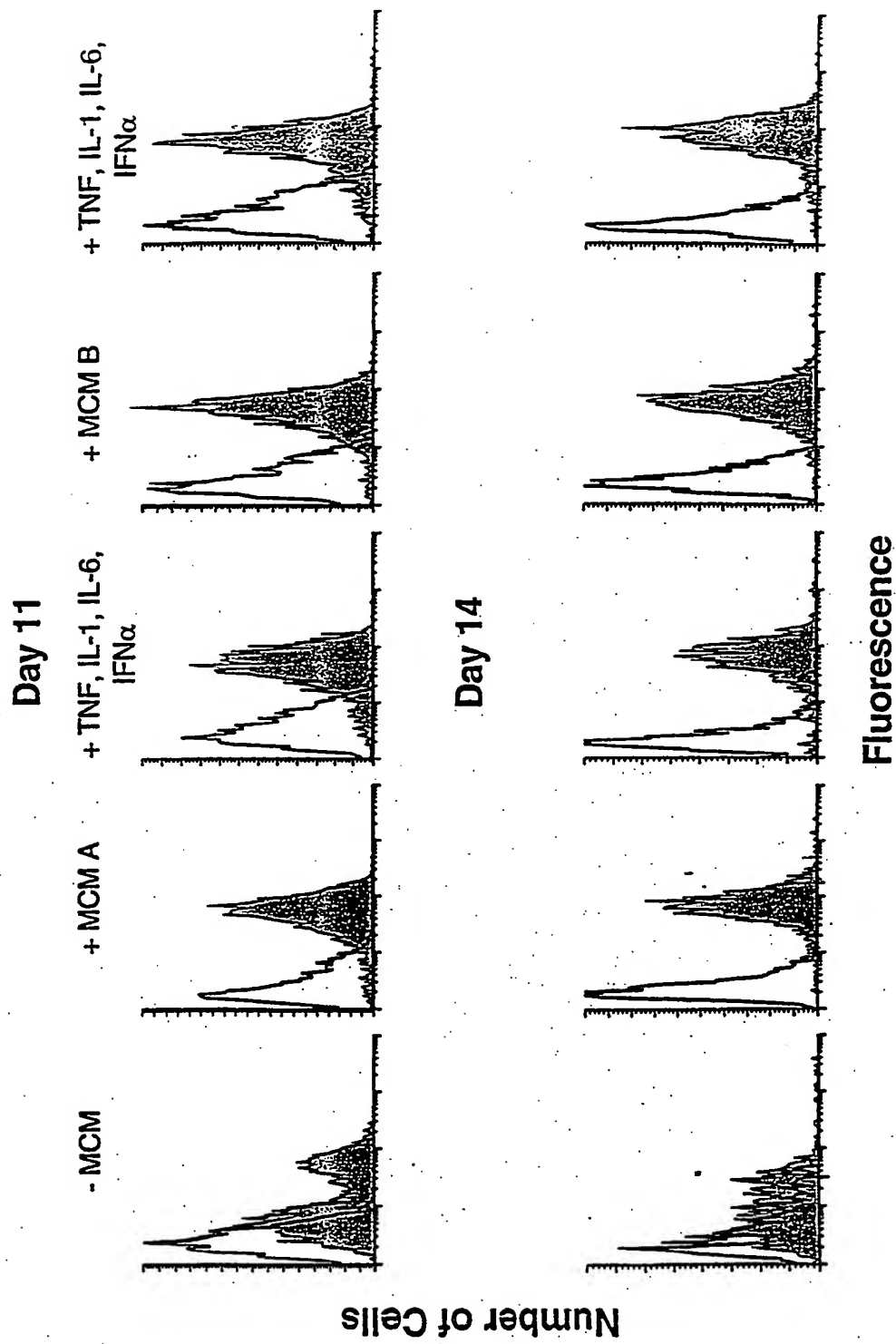


FIG. 14



**FIG. 15**

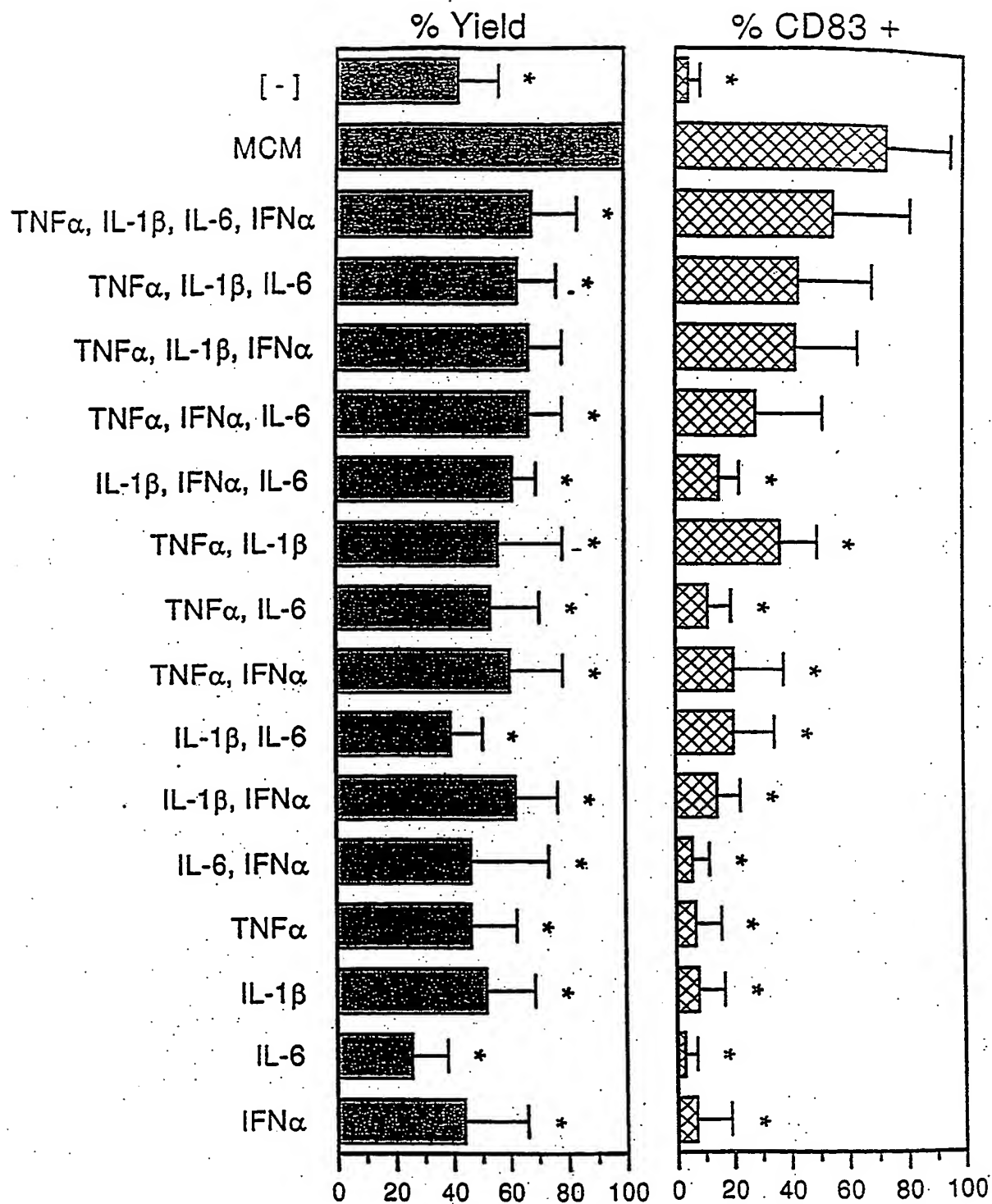


FIG. 16